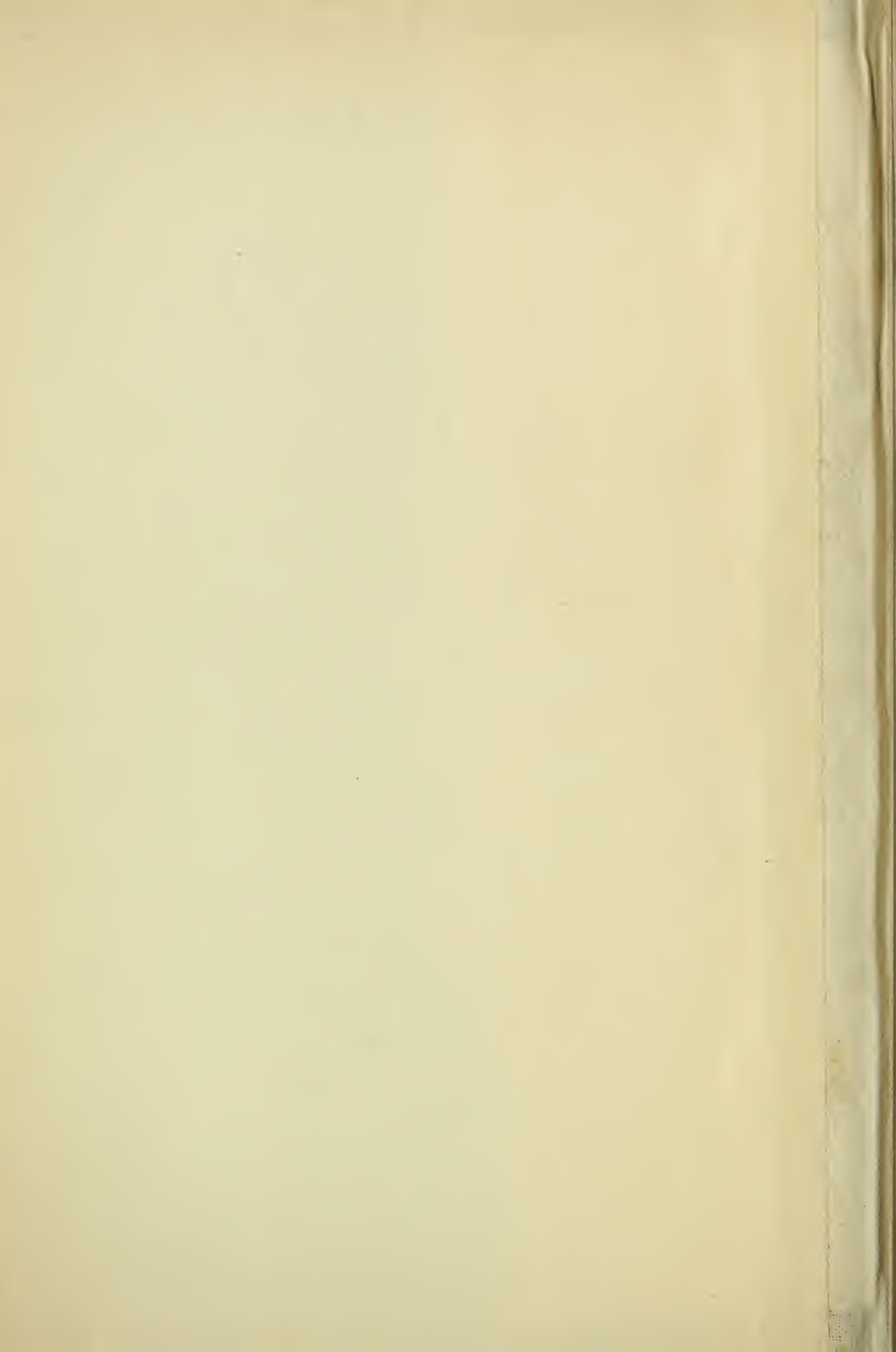


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EDITED BY
SIMON FLEXNER, M.D.

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EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VI. IMMUNITY REACTIONS.

By PETER K. OLITSKY, M.D., AND FREDERICK L. GATES, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 16, 1921.)

In the earlier experiments of this series in which the anaerobic, filter-passing microorganism, *Bacterium pneumosintes*, was transmitted from the nasopharyngeal secretions of influenza patients to the respiratory tract of rabbits, and passed from rabbit to rabbit through many generations, it was our practice to inoculate two or more rabbits in each experiment. One or more of these animals was killed after 24 to 48 hours, as already described,¹ for study and to obtain material for further inoculation. The infection, however, was permitted to run its course in a number of rabbits, which upon recovery became available for subsequent studies in immunity.

The experiments made with the latter rabbits may be grouped according to the nature of the injected material (Table I). These animals were reinjected after varying intervals with material from other sources. The tests were particularly useful in establishing the identity of the active agent derived from human nasopharyngeal washings, and from the lung tissues of affected rabbits, with the cultures of *Bacterium pneumosintes*.

A single typical protocol of the experiments will serve to show the method followed and the results obtained.

Group A. Influenzal Nasopharyngeal Washings versus Active Agent in Lungs of Affected Rabbits.

Experiment 1.—Rabbit A for 4 days prior to injection showed an average leucocyte count of 11,821, of which 6,190 were mononuclears, and temperature of 39.6°C. Mar. 29, 1919. Inoculated intratracheally with 3 cc. of the unfiltered nasopharyngeal washings from Case 16.¹ Mar. 30. Conjunctivitis. Total

¹ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

leucocytes 9,000, of which 3,600 were mononuclears. Temperature 39.6°C. Mar. 31. Conjunctivitis. Total leucocytes 7,025, of which 1,967 were mononuclears. Temperature 40°C. Apr. 1. Conjunctivitis. Total leucocytes 6,325, of which 2,404 were mononuclears. Temperature 40°C. Apr. 2. Recovered. Total leucocytes 11,500, of which 4,140 were mononuclears. Temperature 39.4°C. The animal remained well until Apr. 21, 1919, when it was reinjected, intratracheally, with 3 cc. of lung tissue suspension from a rabbit representing the fifth passage of the nasopharyngeal secretions from Case 17.¹ This active material had been filtered between the third and fourth rabbit passages. No observable effects on the rabbit's appearance, behavior, blood count, or temperature resulted.

As a control for the first injection, Rabbit B was injected along with Rabbit A, and with the same material. For 2 days after the injection Rabbit B showed a

TABLE I.
Groups of Immunity Experiments.

Group.	First injection.	Second injection.
A	Nasopharyngeal washings from influenza patients.	Suspensions of lungs of affected rabbits.
B	Suspensions of lungs of affected rabbits.	Similar suspensions from another animal.
C	Washed mass cultures of <i>Bacterium pneumosintes</i> .	Similar cultures of another strain.
D	Suspensions of lungs of affected rabbits.	Washed mass cultures of <i>Bacterium pneumosintes</i> .
E	Washed mass cultures of <i>Bacterium pneumosintes</i> .	Suspensions of lungs of affected rabbits.

typical clinical picture, with a fall in the leucocyte count, affecting especially the mononuclears, and a rise in temperature. The animal was then killed and autopsy revealed the characteristic hemorrhagic edema and emphysema of the lungs, without involvement of other organs.¹ Similarly, Rabbit C served as a control for the second injection of Rabbit A, and showed typical clinical and pathological effects.

This experiment, typical of three in this series, indicates the development of a protective resistance to the active agent in the rabbit passage experiments, as the result of the previous injection of the nasopharyngeal secretions from an early case of influenza. The nasopharyngeal secretions are thus further shown to be the source of the filterable active material of the rabbit passages. In this instance,

the interval between injections was 23 days. In another experiment, the resistance was maintained for 3 months.

Group B. Active Agent in Lungs of Affected Rabbits versus Similar Material Originally Derived from Other Patients.

That the strains of the active agent in lungs of affected rabbits, originally derived from different influenza patients, have the same antigenic properties and thus give rise to protection against each other is brought out by the following experiment.

Experiment 2.—Rabbit A, having a normal leucocyte count of 14,525, of which 6,100 were mononuclears, and a temperature of 39.8°C., was injected intratracheally on Feb. 25, 1919, with 3 cc. of a saline solution suspension of rabbit lung from the fourth passage of the nasopharyngeal secretions of Case 11,¹ obtained during the first influenzal wave of 1918–19. The subsequent train of events carried the mononuclear cells to 1,242, and the temperature to 40.65°C. on the 3rd day. The fever and mononuclear leucopenia persisted on the 4th and 5th days, with recovery on the 6th and 7th days, when the total leucocyte count was 20,450, of which 5,521 were mononuclears, and temperature 39.6°C. Nearly 14 months later, May 11, 1920, this animal was injected with 3 cc. of a suspension of glycerolated lung tissue from the first rabbit inoculated with the nasopharyngeal secretions of Case 26,¹ from the second or 1920 epidemic. The rabbit developed no significant changes in the blood count or temperature, and when killed, on May 13, showed no visible lung lesions.

The control rabbit, B, similarly injected with the same glycerolated lung tissue, developed the typical blood picture and lung lesions, and the presence of the active agent in the lungs was demonstrated by transmission to a second rabbit which reacted in the characteristic manner.

This experiment not only demonstrates the identity of the antigenic characters of the active material derived from the two epidemics of 1918–19 and of 1920, but shows that the immunity conferred by a previous infection with the active agent may last for a long time.

With the isolation of *Bacterium pneumosintes* from the nasopharyngeal secretions of influenza patients and from the lung tissue of affected rabbits, it became possible to study the character of the active agent of the animal passages by means of cross-protection experiments. It was first necessary to show that recovery from infection with *Bacterium pneumosintes* induces resistance to a subsequent inoculation of the same microorganism.

Group C. Bacterium pneumosintes versus the Same Microorganism.

Experiment 3.—Rabbit A, normal leucocyte count 9,600, of which 6,144 were mononuclears. Temperature 39.3°C. Injected intratracheally with 3 cc. of a saline suspension of washed mass culture in the seventh generation of a rabbit lung strain of *Bacterium pneumosintes* from Case 16,¹ a patient from the first (1918–19) epidemic wave. 1st day after injection: total leucocytes 8,400, of which 2,520 were mononuclears; temperature 39.3°C. 2nd day: total leucocytes 3,800, of which 1,444 were mononuclears; temperature 39.6°C. 3rd day: total leucocytes 5,400, of which 2,052 were mononuclears; temperature 39.6°C. 4th day: recovery; total leucocytes 11,600, of which 4,640 were mononuclears; temperature 39.3°C. 5th day: total leucocytes 15,000, of which 7,500 were mononuclears; temperature 39.4°C. On the following day, when the total leucocytes were 13,600, of which 5,440 were mononuclears, and the temperature was 39.8°C., this animal was reinjected with a similar washed mass culture of a strain of *Bacterium pneumosintes* in the seventh generation from the filtered nasopharyngeal secretions of Case 17,¹ from the same epidemic. During the next 5 days the total leucocyte count remained about 13,000, with an increase in the mononuclear cells to an average of 8,292, and the temperature was constant at 39.7°C.

For assurance that the injected mass culture was active and capable of infecting normal rabbits, a control animal was injected, each time, along with Rabbit A, and with the same material. For 48 hours after injection, these rabbits, B and C, showed the characteristic leucocytic depression, involving especially the mononuclears. In each instance they were then killed, and the usual emphysema and hemorrhagic edema of the lungs were found.²

To show that the reactions described were due to *Bacterium pneumosintes* and not to some constituent of the culture medium, Rabbit D was inoculated intratracheally with 3 cc. of a saline suspension of the sediment of uninoculated, sterile, mass culture medium, at the time that Rabbit A was first injected. The animal gave no reaction. At the time of the second injection, Rabbit D was also injected with the seventeenth generation of the culture of the nasopharyngeal secretions of Case 17. This animal promptly developed the clinical and pathological syndrome characteristic of infection with *Bacterium pneumosintes*.²

Group D. Active Agent in Lungs of Affected Rabbits versus Bacterium pneumosintes.

We have already stated² that “the active material, pathogenic for rabbits and guinea pigs, found in the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza has been identified in the anaerobic organism” (*Bacterium pneumosintes*).

² Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

Additional evidence in support of this statement is furnished by the results of cross-protection experiments of which the following are examples.

Experiment 4.—Rabbit A, normal leucocyte count, 9,200, of which 6,072 were mononuclears. Temperature 39°C. Injected intratracheally with 3 cc. of a saline solution suspension of rabbit lung tissue representing the fifth passage of the filtered nasopharyngeal secretions from Case 26.¹ The following day, leucocytes were 6,400, of which 2,560 were mononuclears. Temperature 39.3°C. 2nd day: leucocytes 7,200, of which 2,763 were mononuclears; temperature 39.4°C. 3rd day: leucocytes 4,400, of which 1,760 were mononuclears; temperature 39.4°C. 4th day: recovery with leucocytes 11,000, of which 6,600 were mononuclears; temperature 39.2°C. 15 days later this rabbit was injected intratracheally with 3 cc. of a saline solution suspension of washed mass culture of *Bacterium pneumosintes*, second generation, originally derived from the nasopharyngeal secretions of the same patient. On the 2 days following, the total leucocyte counts were 14,600 and 16,800, of which 7,800 and 9,240 were mononuclears; temperature 39.5°C. Killed. Autopsy revealed no visible lesions in the lungs. The activity of the materials used in these injections was demonstrated by the injection of control rabbits, B and C, both of which gave typical reactions. In similar experiments, the reinjected rabbits were observed for several days after the second injection to detect any delayed effects. They remained normal.

In Experiment 4, the interval between the first and second injections was 15 days. In three other experiments, identical results were obtained after 3 weeks, and in a fifth, after 9 weeks. In a sixth experiment, involving an interval of 18 days, the second injection produced the typical clinical and pathological reaction. That such protection experiments should occasionally be unsuccessful is not surprising, in view of the arbitrary dosage employed.

Group E. Bacterium pneumosintes versus Active Agent in Lungs of Affected Rabbits.

The converse of Experiment 4 was performed in a series in which *Bacterium pneumosintes* was injected first and the animals were later exposed to the influenzal active agent by the intratracheal injection of affected rabbit lung.

Experiment 5.—Rabbit A showed a normal leucocyte count of 12,000, of which 6,240 were mononuclears. Temperature 39°C. It was injected intratracheally with 3 cc. of a saline solution suspension of washed sediment from a mass culture

of *Bacterium pneumosintes*. During the following 3 days, the total leucocyte count averaged 7,200, of which 2,200 were mononuclears, and the temperature rose to 39.6°C. Thereafter the animal returned to normal. 2 weeks after the first injection, this rabbit was inoculated intratracheally with 3 cc. of a suspension of the active agent in rabbit lung tissue from the fifth passage of the nasopharyngeal secretions of Case 26.¹ After waiting for 48 hours, during which time no change occurred in the blood or temperature, the rabbit was killed. The lungs appeared normal.

In three other similar experiments the rabbits were observed for several days after the second injection, and remained normal. The interval between injections in this series was 11 days, 2, 7, and 8 weeks. The usual control animals, B and C, demonstrated the activity of the injected materials in each test.

These cross-protection experiments establish the antigenic identity of *Bacterium pneumosintes* with the active agent derived from the nasopharyngeal secretions of patients in the early hours of epidemic influenza.

SUMMARY AND CONCLUSION.

The experiments described furnish additional evidence of the pathogenic character and the virtual identity of the various strains of the active agent derived from the nasopharyngeal secretions of influenzal patients with which the transmission experiments in rabbits have been carried out.

The active material has been shown to be of antigenic nature, so that rabbits are protected from the effects of a second inoculation. The experiments indicate also the antigenic identity of the various strains of the active agent with each other and with *Bacterium pneumosintes*.

Finally, the experiments show that the protection may persist for 14 months which is the longest period yet tested.

THE FREQUENCY OF BACILLUS INFLUENZÆ IN THE NOSE AND THROAT IN ACUTE LOBAR PNEUMONIA.

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(Received for publication, June 15, 1921.)

The writer has for some time been engaged in the study of the flora of the nose and throat both in well persons and in those suffering from infections of the respiratory tract. Especial attention has been given to the presence of *Bacillus influenzae* and pneumococcus, and some of the results obtained have already been published (1).

During the course of these studies, certain observations have been made concerning the frequency of occurrence of influenza bacilli and pneumococci in the nose and throat, and concerning the varieties of influenza bacilli which are present in these regions. It is possible that these findings may have a bearing upon the etiology of lobar pneumonia, and for this reason they are reported here.

Methods.

The throat cultures were made by means of cotton swabs passed over the posterior wall of the pharynx. Two swabs were used in each case—with one a freshly poured oleate hemoglobin agar plate was inoculated, with the other a fresh blood agar plate.

Cultures from the nose were made by means of a cotton swab passed through a sterile speculum in order to avoid contamination from the nasal hairs. Cultures were usually made from the nasal mucous membrane in the region of the middle turbinate, though occasionally a deviated septum prevented the introduction of the swab as far as this region. The swabs were streaked over freshly poured blood agar plates.

Attention was given not only to the occurrence of colonies of pneumococcus and of *Bacillus influenzae* on the various plates, but an

effort was made to determine the type of influenza bacilli present and especially whether bacilli of more than one type could be isolated from a single culture. For this reason, whenever colonies of *Bacillus influenzae* were present, several individual colonies were marked and a secondary culture on a fresh blood agar slant was made from each of the colonies. In the present paper, the occurrence of hemoglobinophilic bacilli producing hemolysis on blood agar plates, the so called Bacillus X, is not discussed and the presence of this organism is not noted. All organisms here described as *Bacillus influenzae* failed to produce hemolysis.

The media used in this study had an initial hydrogen ion concentration of about pH 7.4. The blood agar was prepared by adding sterile defibrinated rabbit blood to melted agar to a concentration of 4 per cent or by adding blood extract, according to Wollstein's (2) method, to a concentration of 2 per cent.

In this connection, it should be mentioned that all solid media employed in the cultivation of *Bacillus influenzae* should be freshly prepared. The addition of a little broth to the surface of old blood agar plates will not give satisfactory results. In the present study, the plates were poured within an hour of use. For making fresh oleate hemoglobin plates, it is well to employ tubes of oleate medium which may be melted as needed. Care must be taken that the blood cells are completely hemolyzed before the oleate agar is tubed. If this is not done, upon remelting the agar a heavy protein precipitate will be formed.

Indole production was tested by Ehrlich's para-dimethylamino-benzaldehyde method. The clear supernatant fluid from 48 hour cultures in blood broth or in Dunham's peptone solution enriched with blood extract was used. Since an occasional strain, which apparently had grown luxuriantly, failed to produce indole on the first test, all tests resulting negatively were repeated at least once.

Gas production was tested by making either stab or shake cultures in 1 per cent dextrose agar to which blood extract had been added. Since the amount of gas produced is never very great, all negative tests were repeated.

The sugar fermentation tests were made with cultures in Dunham's peptone solution enriched with 2 per cent blood extract. All cul-

ture tubes were incubated in a slanted position so as to expose as large a surface of the medium as possible to the air, since acid production has been shown to be more rapid under these conditions. As a routine procedure, dextrose, which 92 per cent of the strains fermented, was used as a control, and saccharose as the differential sugar.

Occurrence of Bacillus influenzae in the Upper Respiratory Tract of Normal Individuals and of Individuals Suffering from Lobar Pneumonia.

During the recent epidemic of influenza in New York in 1918-19 and in the succeeding winters, the writer made many cultures from the throats of normal individuals to determine the presence or absence of *Bacillus influenzae*. During the period 1918-21, cultures were made from the throats of 1,077 normal individuals, and influenza bacilli were demonstrated to be present in 332, or in 30 per cent (Table I).

While cultures have been made from the nose in a much smaller number of normal individuals and then only during the past year, 1920-21, the results indicate that hemoglobinophilic bacilli are rarely found here, even in persons suffering from coryza, laryngitis, etc. (Table II).

In marked contrast with these findings are the results obtained in a series of cultures made from the nose and throat of patients suffering from lobar pneumonia. In thirty-one of these cases, cultures were made from the throat, and in eighteen, or from 58 per cent of the cases, influenza bacilli were isolated (Table I). In thirty-five cases of pneumonia, cultures were also made from the nose and influenza bacilli were isolated from this source in nine, or in 23 per cent (Table II). In certain other cases influenza bacilli were isolated from the sputum when they could not be demonstrated in the cultures from the nose or throat. Among the entire thirty-five cases, influenza bacilli were isolated from at least one of these three sources in thirty, or 85 per cent of the cases. These cultures were made during the winter of 1920-21, 2 years after the severe epidemic of influenza. During this winter few cases of typical influenza occurred in New York. That this great frequency of association of *Bacillus influenzae* with patients suffering from pneumonia

is not directly related to the epidemic is further rendered probable by the fact that during the 5 years previous to the epidemic, namely from 1913-18, influenza bacilli were isolated from the sputum of 20 per cent of 672 cases of pneumonia studied in the Hospital of The Rockefeller Institute. While the relative number of instances in which influenza bacilli were isolated from this series of pneumonia

TABLE I.

Incidence of B. influenza in Throat Cultures from Normal Individuals and from Individuals Suffering from Lobar Pneumonia.

Date.	Total No. of individuals.	Positive cultures.	Per cent positive.
Normal individuals.			
Winter of 1918-19.....	717	256	35
“ “ 1919-20.....	253	44	17
“ “ 1920-21.....	107	32	29
Total.....	1,077	332	30
Patients with lobar pneumonia.			
1920-21.....	31	18	58

TABLE II.

Incidence of B. influenza in the Nose of Normal Individuals and of Individuals Suffering from Acute Coryza, Laryngitis, Encephalitis Lethargica, and Lobar Pneumonia.

Source of culture.	No. of cases.	Cultures.	B. influenza present.
Normal individuals.....	22	28	—
Acute coryza and laryngitis	13	23	—
Encephalitis lethargica.....	2	2	—
Lobar pneumonia.....	35	35	9

patients is not as great as the percentage of normal persons who in 1918-21 were found to be carrying these organisms, it must be borne in mind that influenza bacilli were only incidentally looked for in the pneumonia patients previous to 1918 and were only brought to our attention by their growth in the mouse into which sputum had been injected. These figures indicate that influenza bacilli

are much more frequently present in the upper respiratory tract of patients with lobar pneumonia than of normal persons, or at any rate can be more frequently isolated. This is especially true of the occurrence of these bacilli in the throat but is also true of their occurrence in the nose.

In searching for influenza bacilli in the nose, we have also noted the presence or absence of pneumococcus. In the cultures from the nose of the twenty-two normal individuals mentioned in Table II, it was possible to isolate pneumococcus in only one instance. More surprising were the results obtained from nasal cultures from the thirteen cases of coryza and laryngitis and from the two cases of encephalitis lethargica. In these instances no pneumococci were found. Park (3), Allen (4), and others have previously shown that cultures from the nose of normal individuals usually yield a very scanty growth of bacteria. Not infrequently cultures from this region have been found to be sterile.

On the other hand, we have made cultures from the nose in thirty-five cases of lobar pneumonia, and in fifteen instances pneumococci were demonstrated to be present. In the majority of instances, the type of pneumococcus isolated was determined and the organism was found to be identical with those associated with the lung lesion as shown by the sputum determination.

Types of Bacillus influenzae in Nose, Sputum, and Throat Cultures from Individuals Suffering from Lobar Pneumonia.

In the classification of the influenza bacilli, use was made of the grouping devised by Stillman and Bourn (5). The following chart indicates briefly the method employed and the mode of identifying the various groups.

	Group.					
	A.	B.	C.	D.	E.	F.
Indole.....	+	—	—	—	+	—
Gas.....	—	+	—	—	—	+
Saccharose.....	—	+	+	—	+	—

From a study of the hemoglobinophilic bacilli isolated, it soon became evident that the organisms present in any one case or even in any one culture were not all of the same type. It was therefore impossible to group the cases according to the presence of *Bacillus*

influenzæ of the different types or even of the prevailing type. A study was made, therefore, of all the strains isolated from cases of lobar pneumonia during the winter of 1920-21 and the results are shown in Table III.

It is evident from this table that the strains of *Bacillus influenza* isolated from the nose, throat, and sputum of patients with lobar pneumonia do not belong in any one group, but that representatives of all groups occur, though the strains belonging in Group A are in the majority.

Of still greater importance than this, however, is the fact that in certain instances the study of several colonies from the same culture

TABLE III.

A Biological Classification of 140 Strains of B. influenza Recovered from the Throat, Sputum, and Nose of Thirty-five Individuals Suffering from Lobar Pneumonia.

Source of culture.	Total No. of cultures.	No. of strains studied.	Group.					
			A.	B.	C.	D.	E.	F.
Throat.....	32	91	32	18	7	32	2	—
Sputum <i>via</i> mouse.....	8	18	8	—	2	8	—	—
Nasal.....	35	31	18	2	1	10	—	—
Total.....	75	140	58	20	10	50	2	—

B. influenza present in 30 cases.
“ “ absent “ 5 “
Total 35 “

plate from the nose or throat has indicated that influenza bacilli of several different groups may be simultaneously present. For instance, in one case from a single plate made from the throat, of five colonies of *Bacillus influenza* studied, two colonies were shown to consist of influenza bacilli of Group C, and three of influenza bacilli of Group D, and in another case three colonies from a throat culture plate were studied and the bacilli from each of the colonies were found to belong in a different group.

Of seven cases of lobar pneumonia in which both nose and throat cultures yielded *Bacillus influenza*, in three a single type of organism was recovered from both nose and throat. In four instances, different type strains were recovered from these two sources.

DISCUSSION.

At the present time, the significance of the above findings is not definite, though certain explanations suggest themselves. Evidence has been accumulating which indicates that the mere presence of pathogenic pneumococci in the upper respiratory tract, or even in the lungs, is in the great majority of cases, possibly in all, not sufficient in itself to initiate the disease lobar pneumonia in man. It is true that Blake and Cecil (6) have shown that pneumonia may be induced in monkeys merely by the introduction of virulent pneumococci into the trachea, but the amount of culture employed was of considerable size, and in any case, in natural infection in man some preceding event would have to be postulated which would make it possible for the organisms to reach the trachea in sufficient numbers to initiate the disease. Furthermore, Blake and Cecil found it difficult to transmit Type I pneumonia from monkey to monkey by intimate and prolonged contact alone, only one out of six attempts being successful. They were also unable to initiate pneumonia in monkeys by the instillation of large amounts of a culture of virulent pneumococcus into the nose and throat, though the monkeys carried pneumococcus in the mouth for a month. From these observations, it seems likely that the mere presence of pneumococci in the normal upper respiratory tract, or even in the lung, can rarely, if ever, be held accountable for the onset of pneumonia.

More and more attention is therefore being given to the factors which are concerned in etiology previous to actual infection by the pneumococcus. It has been shown that 40 per cent of the cases of lobar pneumonia give a history of coryza (7) or other mild infection of the respiratory tract preceding the onset of pneumonia. It seems quite possible that a variety of contributing factors at present unknown, such as mild unrecognized infections, may act in lowering the local or general bodily resistance so that the pneumococcus may invade and infect the lungs.

Our observation that *Bacillus influenzae* and pneumococci are frequently present in the nose and throat of pneumonia cases therefore suggests that these organisms may have been responsible for a coryza or infection of the mucous membranes of the nasopharynx preliminary

to the infection of the lung with the pneumococcus. It must be borne in mind, however, that the relation of these organisms to ordinary colds is at present purely hypothetical. In any case, our observations indicate that in coryza and laryngitis, it is unusual for these organisms to be present in the nose.

Another possible explanation is that during the course of acute lobar pneumonia, the entire mucous membrane of the respiratory tract loses certain properties antagonistic to the growth of microorganisms, and that under these conditions all varieties of bacteria which normally grow sporadically are able to grow with great vigor. At present, however, the exact significance of our observation cannot be established and the above possibilities are offered merely as suggestions.

The fact that the hemoglobinophilic bacilli found in the upper respiratory tract of a given individual under the conditions discussed are not all of the same variety, is at first confusing and difficult to understand. One should bear in mind, however, the conditions in regard to the pneumococcus about which knowledge has been greatly increased during recent years. It is well known that pneumococci are of several types. Epidemiological evidence indicates that the Type IV pneumococci are relatively harmless and are normal inhabitants of the upper respiratory tract, while Type I and Type II pneumococci are almost always found in association with disease. It is not infrequent to find two types of pneumococci in one mouth, especially in the mouths of persons who have been in close association with pneumonia patients, but up to the present time the writer has never encountered a Type I and a Type II pneumococcus in the mouth at the same time. When pneumococci of one of these two types are present, together with pneumococci of Type IV, it is probable that the latter were present before the advent of the Type I or Type II pneumococci. Furthermore, the work of Olmstead (8) indicates that Type IV pneumococci may be further subdivided into definite types. Whether all the pneumococci of Type IV which are found in a single mouth are identical or not is not certain. It is not at all impossible that they may be of different kinds.

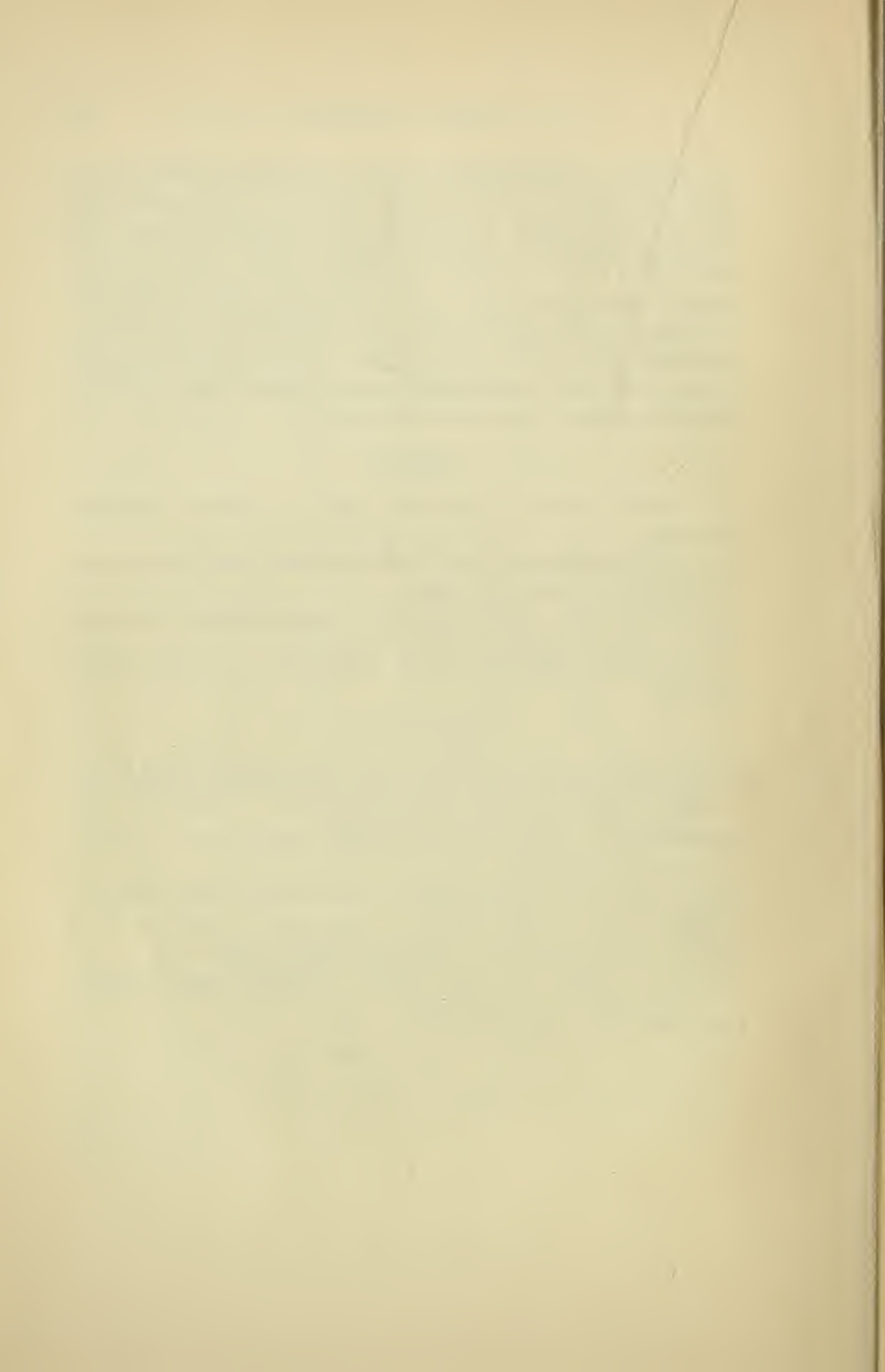
As regards hemoglobinophilic bacilli, the methods which have so far been proposed for separating them into groups or types cannot be said to be satisfactory. It is not possible at present to separate the saprophytic from the disease-producing varieties. The tentative classification based on indole production, gas formation, and saccharose fermentation which we have proposed may not be based on fundamental differences in the bacteria. When further knowledge concerning this group is obtained, the presence of several varieties in one mouth may possibly be shown to depend upon conditions analogous to those relating to pneumococcus.

SUMMARY.

1. *Bacillus influenzae* is frequently found in association with lobar pneumonia.
2. In lobar pneumonia both *Bacillus influenzae* and pneumococcus are frequently found in the nose.
3. The influenza bacilli found in the upper respiratory tract of cases of lobar pneumonia are of various types.
4. The exact significance of these findings is at the present time not clear.

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HETEROGENIC SERUM, AGE, AND MULTIPLICATION OF FIBROBLASTS.

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I.

INTRODUCTION.

It has been shown that the inhibiting action of homogenic serum on the growth of a pure culture of chicken fibroblasts is directly proportional to the age of the animal from which the serum is taken, and that the rate of cell multiplication can be used as a reagent of certain changes produced by age in the blood serum.¹ Similar effects probably could be obtained from fibroblasts of mammals cultivated in homogenic serum. But it would be of advantage to cultivate chicken tissue in heterogenic serum because it is much more difficult to keep alive *in vitro* permanently a strain of mammal fibroblasts than of chicken fibroblasts. Over 10 years ago we found that chick embryo tissues grew abundantly in plasma from rabbits, dogs, and human beings, although not quite as well as in chicken plasma.² Later the effect of many heterogenic sera was investigated. Lambert and Hanes³ cultivated rat sarcoma in guinea pig plasma and Ingebrigtsen⁴ studied the action of several heterogenic sera on cultures of guinea pig bone marrow. The growth was always less rapid and extensive than in homogenic serum, and the toxic action of the serum varied according to the species of the animal. Owing to the lack of accuracy of the technique used at the time, no precise knowledge was gained of the action of these different sera.

¹ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

² Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiv, 244.

³ Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, xiv, 129.

⁴ Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 397; xvi, 421.

A quantitative study of the influence of heterogenic sera, taken from animals of different ages, on the rate of growth of pure cultures of fibroblasts was begun by one of us in 1919. Fragments of a 7 year old strain of chicken fibroblasts were cultivated in media made of one volume of plasma and two volumes of serum taken from cats 1 month, 1 year, and 9 years old. The amount of growth obtained in the kitten serum was about four times greater than in the old cat serum. Similar experiments were made with serum obtained from human beings 22, 40, and 45 years old. Differences in the rate of growth were observed, although they were less marked than in cat serum. It became evident that the growth of chicken fibroblasts was more active in the serum of the young than in that of the old animals. When the technical improvements recently described by Ebeling rendered it possible to cultivate tissues in a medium composed of serum and fibrinogen,⁵ and to prepare the cultures and measure the rate of growth with greater accuracy,⁶ the study of the action of heterogenic serum was resumed. The purpose of the experiments described in this paper was to investigate the relation existing between the rate of growth of a pure culture of fibroblasts and the concentration in the medium of heterogenic serum, and the influence of the age of the animals from which the heterogenic serum was taken on the cell multiplication, in order to ascertain whether a pure culture of chicken fibroblasts could be used as a reagent for the detection of the modifications brought about by age in the blood of mammals.

II.

Action of Various Concentrations of Heterogenic Serum.

The action of different dilutions of homogenic and heterogenic sera on the rate of growth of chicken fibroblasts was thus investigated. The fibroblasts were taken from stock cultures of a 9 year old strain of chicken connective tissue, cultivated in one volume of embryonic tissue juice to two volumes of chicken plasma. Each fragment was divided in two equal parts, one being placed in the experimental medium and the other in the control. The experimental

⁵ Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

⁶ Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

medium was composed of 20 per cent fibrinogen suspension, 5 per cent embryo juice, and varied amounts of Tyrode solution and dog or cat serum. The control medium had a similar composition, but the heterogenic serum was replaced by chicken serum. The fibrinogen suspension was prepared according to the method described by Ebeling.⁵ The embryo juice was obtained from 8 or 9 day old chick embryos. Serum was taken from the plasma of cats or dogs under 2 years of age.

The action of media containing from 5 to 65 per cent heterogenic serum was compared with that of media containing identical amounts of chicken serum. Three cultures were made in each concentration of heterogenic serum. They were controlled by three cultures made in similar concentrations of chicken serum. The results of the experiments, showing the action of dog serum, were expressed by the relative increase of the tissues in 48 hours and are recorded in Tables I and II. The three experiments and controls of each series were averaged and the ratio,
$$\frac{\text{Relative increase in dog serum}}{\text{Relative increase in chicken serum}},$$
 was calcu-

lated. The variations in the rate of growth were expressed by a graph in which the ratio was plotted in ordinates and the serum concentration in abscissæ (Text-figs. 1 and 2). The rate of growth did not differ markedly from the control as long as the concentration of heterogenic sera was lower than 20 per cent. When the concentration of dog serum was increased from 20 to 35 per cent, the inhibiting action of the medium rapidly became more marked, and no growth was observed when the concentration reached 35 to 40 per cent. Other experiments showed that according to the individual, the serum concentration which prevents growth may vary from 30 to 45 per cent. The ratio of both relative increases in dog and chicken sera expressed the inhibiting action of dog serum on the proliferation of the fibroblasts, and can be called growth index of the serum. The growth index of the dog sera for a concentration of 30 per cent was respectively 0.32 and 0.067 (Tables I and II). The growth index of the cat sera for the same concentration (30 per cent) was 0.83 and 0.84. In other experiments, the growth index was also calculated in comparing the relative increases of a pure culture of fibroblasts in dog serum and in Tyrode solution, instead of chicken

TABLE I.
Experiment 1. Influence of Various Concentrations of Dog and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.											
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.	
		Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.
1	21864	4.25	4.9										
2	21865	6.5	6.0										
3	21866	6.3	6.06										
4	21799			4.42	2.78								
5	21800			5.54	2.52								
6	21801			3.8	2.46								
7	21880			4.8	4.2								
8	21881			4.7	4.67								
9	21882			5.6	4.9								
10	21930					3.6	3.5						
11	21931					3.5	2.9						
12	21932					3.8	3.3						
13	21769							2.33	3.04				
14	21928							4.2	3.05				
15	21929							3.4	2.2				
16	21950							3.7	3.5				
17	21951							3.16	2.65				

18	21912								3.0	2.1								
19	21913								3.3	2.3								
20	21914								3.6	1.7								
21	21802										5.0	2.95						
22	21803										3.7	0.95						
23	21804										4.23	0.51						
24	21861												3.3	0				
25	21862												3.58	0				
26	21863												3.6	0				
27	21770															2.7	0	
28	21910															5.5	0	
29	21911															3.7	0	
Average		5.68	5.65	4.81	3.59	3.63	3.2	3.36	2.88	3.3	2.0	4.31	1.47	3.49	0	3.96	0	
Relative increase in dog serum																		
Relative increase in chicken serum		0.96		0.75		0.89		0.93		0.62		0.32		0			0	

16	22015									3.80	0.23	
17	22016									3.00	0.25	
18	22017									3.40	0.21	
19	22036										4.1	0.06
20	22037										3.6	0
21	22038										3.45	0
Average		5.66	5.45	4.9	4.7	3.26	3.1	3.2	2.43	3.4	1.2	3.70
Relative increase in dog serum												
Relative increase in chicken serum		0.96		0.96	0.96	0.98	0.75	0.36	0.067			0.005

TABLE III.
Experiment 1. Influence of Various Concentrations of Cat and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.											
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.	
		Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.
1	22052	5.70	5.20										
2	22053	7.80	6.50										
3	22054	5.50	4.90										
4	22055			5.00	4.90								
5	22056			4.70	4.70								
6	22057			5.60	5.50								
7	22063					4.26	4.00						
8	22064					4.70	4.56						
9	22065					4.50	4.70						
10	22066							4.35	4.80				
11	22067							5.05	5.20				
12	22068							5.50	5.40				
13	22084									3.68	3.65		
14	22085									4.19	4.00		
15	22086									4.20	4.14		

[illegible]

*The results obtained in higher dilutions were not recorded on account of errors due to liquefaction of the medium.

TABLE IV.

Experiment 2. Influence of Various Concentrations of Cat and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.																								
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.		Serum concentration 40 per cent.		Serum concentration 45 per cent.		Serum concentration 50 per cent.		Serum concentration 55 per cent.		Serum concentration 60 per cent.		
		Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	
1	22242-1 & 2	5.58	5.06																						Chicken serum.	Cat serum.
2	22243-1 & 2	4.56	4.69																							
3	22244-1 & 2	4.72	4.87																							
4	22245-1 & 2			4.90	4.70																					
5	22246-1 & 2			3.86	4.90																					
6	22247-1 & 2			4.56	4.44																					
7	22251-1 & 2			3.65	4.24																					
8	22252-1 & 2			3.96	4.24																					
9	22253-1 & 2			3.74	2.13																					
10	22254					4.09	4.26																			
11	22255					4.30	3.18																			
12	22256					3.55	2.83																			
13	22257									3.57	3.02															
14	22258									3.57	3.24															
15	22259									3.00	2.83															
16	22266																									3.85 3.18
17	22267																									4.14 3.26
18	22268																									3.84 3.48

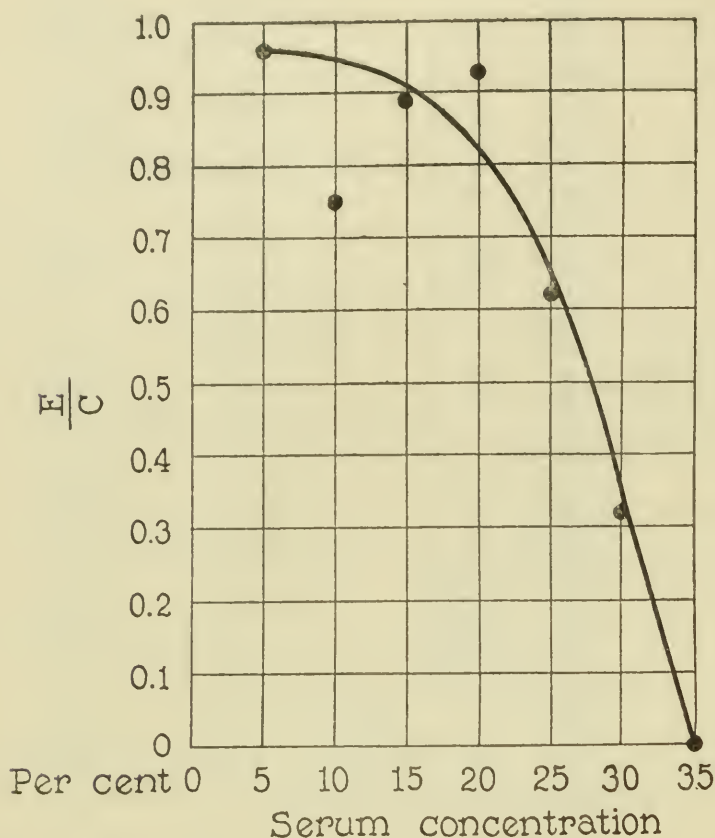
TABLE V.

Influence of Various Concentrations of Young and Old Cat Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.											
		Serum concentration 5 per cent.	Serum concentration 10 per cent.	Serum concentration 15 per cent.	Serum concentration 20 per cent.	Serum concentration 25 per cent.	Serum concentration 30 per cent.	Serum concentration 35 per cent.	Serum concentration 40 per cent.	Serum concentration 45 per cent.	Serum concentration 50 per cent.	Serum concentration 55 per cent.	
		Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.
1	22299-1 & 2	5.30	4.83										
2	22300-1 & 2	4.06	3.88										
3	22301-1 & 2	3.61	3.47										
4	22302-1 & 2			4.70	4.08								
5	22303-1 & 2			4.33	4.58								
6	22304-1 & 2			4.80	4.60								
7	22359-1 & 2			5.47	4.70								
8	22360-1 & 2			4.35	3.88								
9	22361-1 & 2			4.75	4.21								
10	22362-1 & 2					4.32	4.67						
11	22363-1 & 2					5.02	4.56						
12	22364-1 & 2					5.12	4.06						
13	22365-1 & 2					4.93	4.65						
14	22366-1 & 2					4.80	4.12						
15	22367-1 & 2					4.15	2.98						
16	22382-1 & 2											3.52	2.94
17	22383-1 & 2											3.66	2.98
18	22384-1 & 2											3.10	2.56

[illegible]

serum. Similar experiments were made with cat serum and their results recorded in Tables III and IV, and in Text-figs. 3 and 4. The action of cat serum was not apparent until a concentration of 20 to 25 per cent was reached. Afterward, its action became stronger and no growth was observed when the concentration had reached 60 per cent.



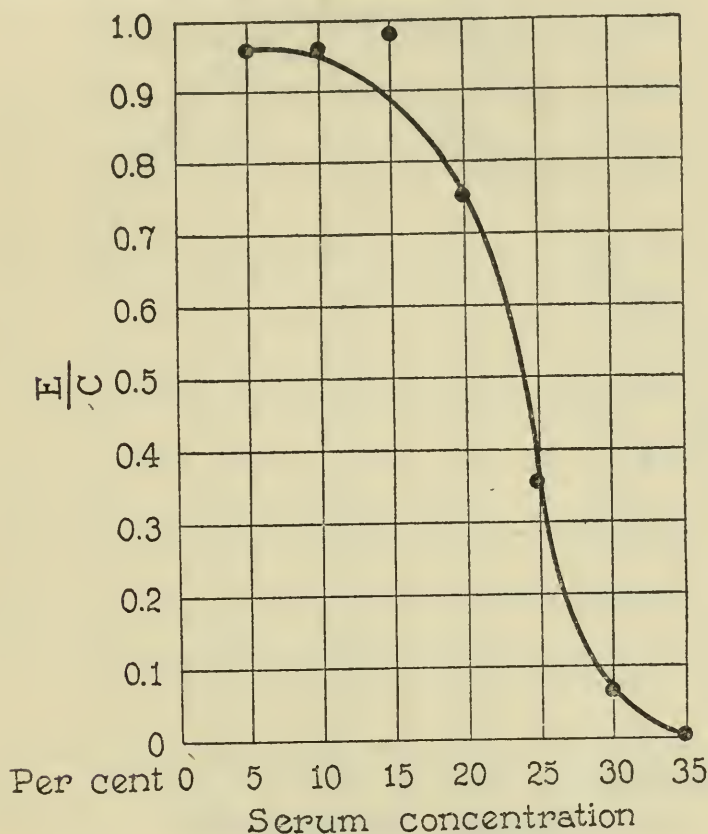
TEXT-FIG. 1. Experiment 1. Relation between the rate of growth of fibroblasts in dog and chicken sera. The ratio, $\frac{\text{Relative increase in dog serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

III.

Action of Heterogenic Sera from Young and Old Animals.

An examination was then made as to whether heterogenic sera from young and old animals would differ in their action on the growth of fibroblasts. Sera were obtained from cats 6 months and 9 years of age, and from dogs 6 months and about 10 years old. The tech-

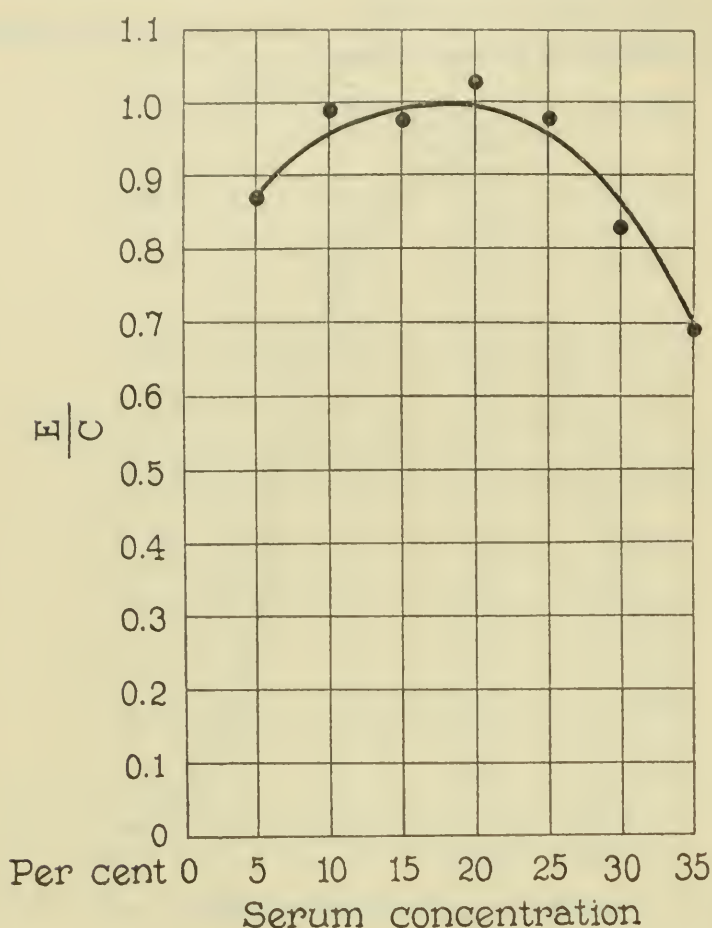
nique was identical with that previously described. The relative increases of the cultures of fibroblasts in young and old cat sera were compared. In Table V are recorded the relative increase of the tissue in both media, and the quotient of the figures expressing the rate of multiplication in the old and young cat sera. In Text-fig. 5, the ratio, $\frac{\text{Relative increase in old serum}}{\text{Relative increase in young serum}}$, was plotted in ordinates and



TEXT-FIG. 2. Experiment 2. Relation between the rate of growth of fibroblasts in dog and chicken sera. The ratio, $\frac{\text{Relative increase in dog serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

the serum concentration in abscissæ. The graph shows that the action of both sera was about the same as long as the concentration present in the medium was not above 20 per cent. Afterward, the growth decreased more rapidly in the old animal serum. When the concentration reached 50 per cent, the rate of growth was only 32 per cent of that observed in a medium containing a similar amount of young cat serum.

The same experiments were repeated with sera obtained from a dog 6 months old, and from another about 10 years old. The relative increase of the cultures of fibroblasts in both sera is recorded in Table VI and Text-fig. 6, in which the ordinates represented the quotient of the rates of growth in old and young sera, and the abscissæ



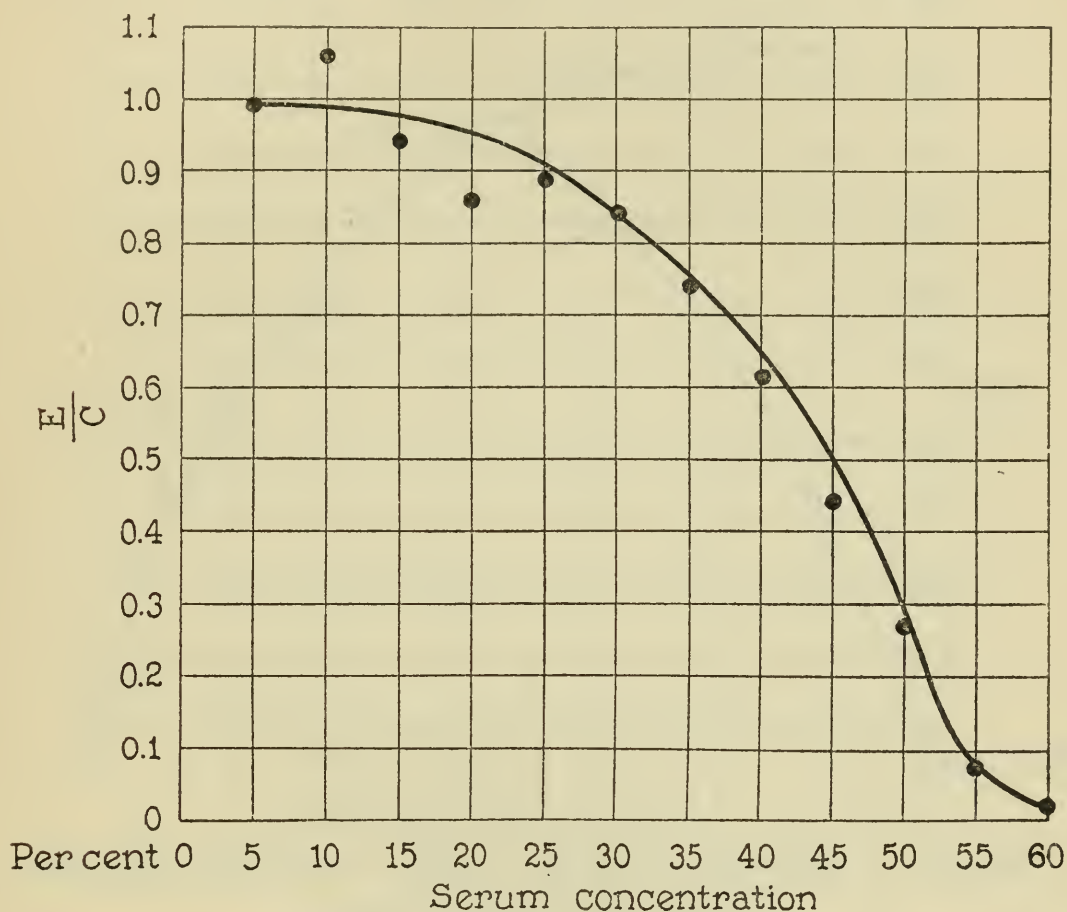
TEXT-FIG. 3. Experiment 1. Relation between the rate of growth of fibroblasts in cat and chicken sera. The ratio, $\frac{\text{Relative increase in cat serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

the serum concentration in the medium. The graph shows that the inhibiting action of the old dog serum was more marked than that of the young dog serum. When the concentration of the serum reached 40 per cent, the rate of growth in the serum of the old animal was only 23 per cent of the rate of growth in the serum of the young one.

IV.

SUMMARY AND CONCLUSIONS.

The presence in a culture medium of heterogenic serum of various concentrations exerts a definite influence on the rate of multiplication of fibroblasts. Dog serum does not inhibit the growth of

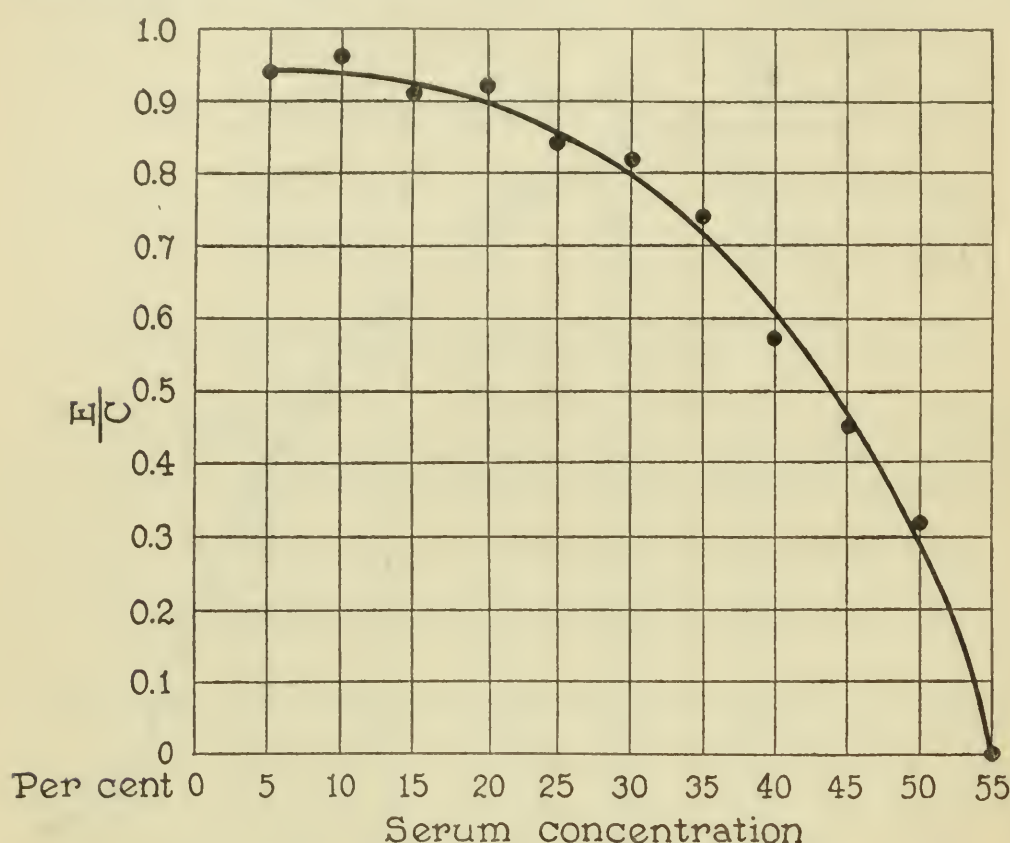


TEXT-FIG. 4. Experiment 2. Relation between the rate of growth of fibroblasts in cat and chicken sera. The ratio, $\frac{\text{Relative increase in cat serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

chicken fibroblasts markedly until its concentration reaches 15 per cent. Beyond this figure, each increase of the concentration brings about a rapid decrease in the rate of cell multiplication. When the concentration reaches from 30 to 45 per cent, no growth takes place. The inhibiting action of cat serum begins to manifest itself at a concentration of 25 per cent and prevents cell prolifera-

tion completely at a concentration of 55 and 60 per cent. The ratio, $\frac{\text{Rate of growth in heterogenic serum}}{\text{Rate of growth in chicken serum}}$, can be taken as expressing

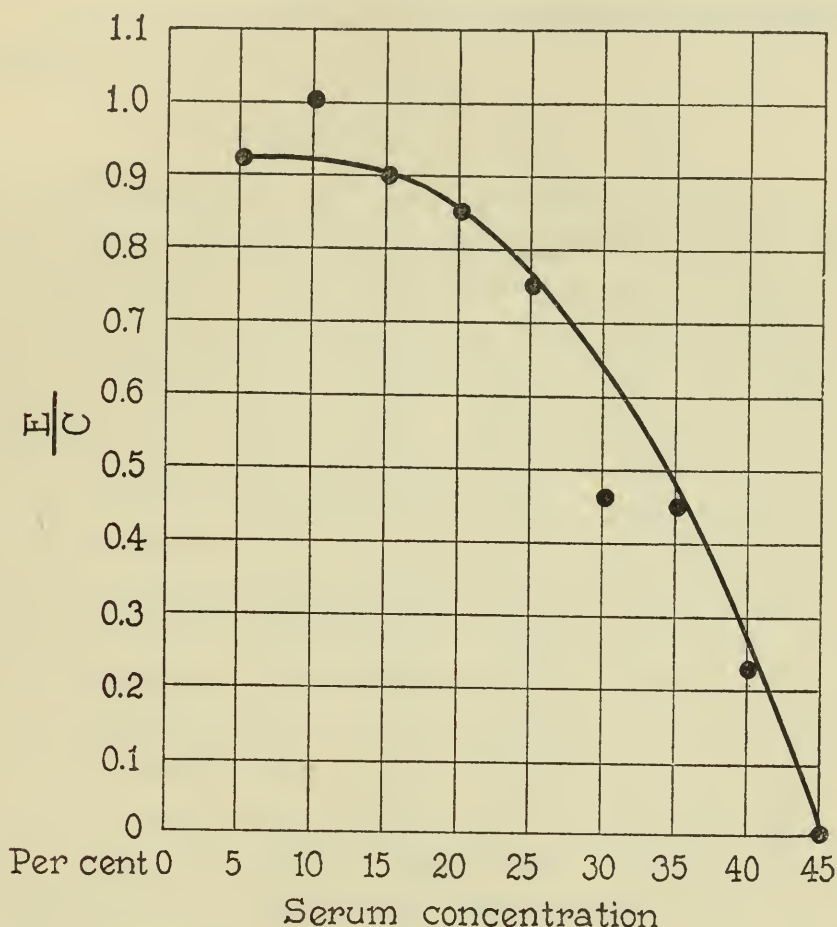
the action of the serum on fibroblast multiplication; that is, as the growth index of the serum.



TEXT-FIG. 5. Relation between the rate of growth of fibroblasts in young and old cat sera. The ratio, $\frac{\text{Relative increase in old serum}}{\text{Relative increase in young serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

The inhibiting influence of heterogenic serum was found to vary in direct ratio to the age of the animal from which it was obtained. The rate of proliferation of chicken fibroblasts was studied comparatively in media containing varied concentrations of serum from young and old animals. For each concentration of serum, the rate of growth in the serum of the old animal was expressed in relation to the rate of growth in the serum of the young animal. When cat

serum was used, the curve obtained in plotting this ratio in ordinates and the serum concentration in abscissæ showed a rapid increase in the inhibiting action of the old serum as soon as the concentration reached 30 per cent. The same tests were repeated with the serum from young and old dogs. The general results were identical, although



TEXT-FIG. 6. Relation between the rate of growth of fibroblasts, in young and old dog sera. The ratio, $\frac{\text{Relative increase in old serum}}{\text{Relative increase in young serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

the quantitative inhibiting action of both sera was greater than that of cat serum. It may be concluded that under the conditions of the experiments:

1. Heterogenic sera inhibit and prevent the growth of chicken fibroblasts when their concentration is made to vary within certain limits.

2. A relation exists between the rate of growth of chicken fibroblasts, the concentration of the heterogenic serum, and the age of the animal from which the serum is taken. Thus, the rate of proliferation of chicken fibroblasts expressed by the growth index of the serum can be used as a means for detecting certain changes brought about by age in a heterogenic serum.

A STUDY OF THE RELATION OF *TREPONEMA PALLIDUM* TO LYMPHOID TISSUES IN EXPERIMENTAL SYPHILIS.

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The establishment of the fact that dissemination of *Treponema pallidum* in the rabbit occurs after local inoculation has for the most part rested upon the evidence of the development of syphilitic lesions remote from the site of the primary lesion and upon occasional demonstrations of spirochetes in certain tissues, such as the spleen, blood, bone marrow, and lymph nodes. Our experience of the frequency of generalized lesions following inoculation of testicle or scrotum has differed from that of most workers on experimental syphilis, and in addition we have shown that it is possible to produce generalized lesions with great regularity by simple technical procedures either at the time of infection or shortly thereafter so that under certain conditions at least, dissemination of *Treponema pallidum* is known to have occurred in every animal infected (1). The mechanism of the process, however, is still undetermined, and before the question of a generalized infection can be put upon a logical basis, it is necessary that such essential facts as the time, frequency, and extent of dissemination under the usual conditions of inoculation procedure together with the localization and extent of the infection should be obtained. Among the possible modes or paths of dissemination which might be assumed to participate in the process of generalization, are the lymphatic and blood systems, and both are easily accessible to experimental investigation, as indicated in a preliminary communication (2).

Among the generalized manifestations of experimental syphilis in the rabbit, enlargement and induration of various lymph nodes are commonly observed. The superficial nodes which most frequently show various degrees of involvement after the usual scrotal or sheath inoculation are the inguinals, and similar changes are seen in the sacral retroperitoneal glands following testicular inoculations. In-

volvement of other nodes such as the flank, axillary, auricular, cervical, and submental glands has also been observed in certain animals, and apparently some abnormality of the popliteals is a constant feature of the infection irrespective of the site of inoculation.

Such changes as take place, however, might logically be expected to appear first in the lymph nodes draining the area of the primary reaction if dissemination of spirochetes from a local focus of inoculation regularly occurs by way of the lymphatics, and consequently the problem was first approached by a series of experiments designed to show the presence or absence of *Treponema pallidum* in the inguinal lymph nodes of rabbits inoculated in the scrotum.

The studies of the part played by the blood stream in the process of dissemination of *Treponema pallidum* are reserved for a later publication, but it should be stated here that before it could be concluded that organisms were localized in lymphatic tissue, it was necessary to exclude the blood as a possible source of spirochetes. During the early stages of the infection, this could be done only upon the basis of relative infectivity. Later, however, blood inoculations were uniformly negative. It is obvious, moreover, that a blood stream infection must be ruled out before one can conclude that spirochetes are localized in any other tissue, as for instance in the spleen or bone marrow.

EXPERIMENTAL.

The inguinal lymph nodes of normal rabbits are small, flattened or ovoid masses which are barely visible or palpable and are frequently imbedded in relatively large pads of fat which renders them more difficult of detection. Gross changes in the nodes are usually detected within 4 or 5 days after scrotal inoculation and consist in a slight enlargement together with an increased resistance or tension.¹ As the process continues, the glands rapidly become indurated and many times their normal size, and, in our experience, show some degree of abnormality as long as an active syphilitic process exists in the scrotum.

¹ This was the condition at the time these experiments were carried out. Subsequently, however, definite alterations of the nodes have frequently occurred within 24 hours after scrotal or sheath inoculation.

The demonstration of *Treponema pallidum* in lymph nodes may be accomplished by two methods—dark-field examination of fluid aspirated from the node or by animal inoculation. Direct aspiration of lymph nodes *in situ* by means of a syringe is a difficult procedure, particularly in the early involvement of the nodes, and the fluid obtained is often contaminated with blood. Moreover, as will be pointed out later, such a procedure may necessitate the examination of many preparations, and, therefore, we had recourse to the second and more satisfactory method which consisted in the removal of an inguinal node under ether anesthesia, the making of a node emulsion with salt solution, and the injection of this emulsion into the testicles of normal rabbits.

The extirpated lymph node is first minced with fine scissors and then ground in a sterile mortar with a small quantity of sterile normal salt solution. In most of our experiments we used a total quantity of 1.5 cc. of saline solution and were able to recover 1.2 cc. of the emulsion; 0.6 cc. was then injected into one testicle of each of two normal rabbits by means of a Fournier tuberculin syringe and a No. 22 gauge Luer needle. The subinoculated rabbits were closely followed during an observation period of 2 months for the development of syphilitic lesions. The organisms used in these experiments were the Zinsser and the Nichols strains of *Treponema pallidum* which have been maintained in rabbits for periods of 7 to 8 years.

Results.

The Frequency and Time of Inguinal Lymph Node Involvement.

Our preliminary experiments were concerned with the demonstration of the presence of *Treponema pallidum* in the inguinal lymph nodes of a few rabbits with large actively growing chancres. The results of the individual experiments are grouped together, and, as may be seen in Table I, the interval of time after scrotal inoculation at which the six experiments were carried out was progressively shortened from 61 to 16 days. The chancres were all well developed, while the inguinal glands were many times normal size, and markedly indurated. One gland was excised from each rabbit. Although spirochetes were found by dark-field examination in but one gland emulsion

a syphilitic orchitis developed in all the subinoculated rabbits in from 15 to 41 days as a result of the testicular injection of the lymphatic gland emulsion, showing that there had been an actual dis-

TABLE I.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 61 to 16 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	Interval between scrotal inoculation and excision of inguinal lymph node.	Examination of inguinal node emulsion for spirochetes.	Rabbit inoculated with lymph node emulsion.		Result of inoculation.	Incubation period of orchitis.
			No.	Testicle.		
	<i>days</i>					<i>days</i>
A	61	Negative.	1	Right. Left.	+ +	41
B	49	"	2	Right. Left.	+ +	Exact time not known.
C	35	Positive.	3	Right. Left.	+ +	15 33
D	35	Negative.	4	Right. Left.	+ +	33 33
E	18	"	5	Right. Left.	+ +	27 28
			6	Right. Left.	+ +	26 28
F	16	"	7	Right. Left.	+ +	34 34
			8	Right. Left.	+ +	26 28
Total..6						

+ indicates infection.

In the preliminary experiments, one rabbit was used for each gland emulsion and both testicles were inoculated, but later, the precaution of using two rabbits was adopted. In the majority of experiments, one testicle only was inoculated.

semination of spirochetes in each instance from the local focus of infection in the scrotum.

Our next experiment was planned to determine the incidence of infection of the inguinal nodes 7 days after scrotal inoculation, when

enlargement and increased resistance of the nodes are found in practically every instance, although the specific reaction about the implant is usually just beginning. The results of the experiment are shown in Table II.

TABLE II.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 7 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	Rabbit inoculated with lymph node emulsion.		Result of inoculation.	Incubation period of orchitis. <i>days</i>
	No.	Testicle.		
G	9	Right.	+	28
		Left.	+	28
	10	Right.	+	28
		Left.	+	28
H	11*	Right.	+	35
	12*	"	+	33
I	13*	"	+	35
	14*	"	+	35
J	15	"	+	30
	16	Accidental death.		
K	Spirochetes +; no inoculation.			
L	"	+; " "		
Total...6				Average..32

* Animals of poor quality with very small testicles.

+ indicates infection.

A series of six rabbits was operated upon and one of the inguinal glands removed. Dark-field examination of the gland emulsions showed actively motile spirochetes in two instances and no subinoculations were made from these; the remaining four emulsions were injected into the testicles of eight normal rabbits. The injection of one emulsion into both testicles of two rabbits resulted in an acute orchitis which developed simultaneously in 28 days in the four testicles, while the remaining three emulsions were injected into one testicle each of six rabbits. There was one accidental death soon after inoculation, but in the surviving five animals, a syphilitic orchitis developed in from 30 to 35 days and progressed actively in the usual fashion.

The consistently positive results of this experiment were most striking in that all six animals examined 7 days after scrotal inoculation were found to have infected inguinal glands in spite of the fact that a specific reaction about the scrotal implant could scarcely be detected.

In the next experiment, the interval of time between scrotal inoculation and extirpation of the inguinal node was shortened to 5 days (Table III).

The saline emulsions of ten inguinal glands were injected into the testicles of eighteen normal rabbits; in two instances, one subinoculation was made, but in eight, two rabbits were used. One bilateral inoculation was made. Spirochetes were not found by dark-field examination in any of the gland emulsions. A syphilitic orchitis, however, developed in seventeen of the eighteen test rabbits with an average incubation period of 30 days; in two instances, the orchitis was detected 25 days and in five instances, 28 days after inoculation. In sixteen of the eighteen rabbits, the reaction was of a relatively rapid progressive type, while in one rabbit with very small atrophic testicles, it was not particularly active and tended toward a localized area of induration. The orchitis which resulted from the single bilateral inoculation in the experiment developed in 33 days in one testicle and 39 days in the other, which was the longest incubation period in the series. In one animal a progressive orchitis failed to develop during the observation period of 8 weeks, and it is possible that this was an instance of an atrophic orchitis; the companion rabbit, however, inoculated with the same material, showed the usual reaction with an incubation period of 34 days.

The results of this experiment coincide to a remarkable degree with those obtained in the previous series and demonstrate in a considerable number of animals that the inguinal lymph nodes of rabbits inoculated in the scrotum only 5 days previously and at a time when no specific reaction about the implant was observed were constantly infective. In addition, although no spirochetes were demonstrated in the lymph gland emulsion by dark-field examination, the material was sufficiently potent to produce an actively developing syphilitic orchitis with an average incubation period of 30 days.

Finally, a similar experiment was undertaken 2 days after scrotal implantation when no gross alteration of the inguinal nodes was detected by palpation or by inspection, since the previous experiments had shown that enlargement and tenseness of the nodes, 5 and 7 days after scrotal inoculation, were constantly associated with the presence of *Treponema pallidum*.

TABLE III.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 5 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	No. of rabbit inoculated with lymph node emulsion.	Result of inoculation.*	Incubation period of orchitis.
			<i>days</i>
M	17	+	33
	18	+	30
N	19	+	28
O	20†	?	?
	21	+	34
P	22	+	33
Q	23‡	+	33 and 39
	24	+	28
R	25	+	25
	26	+	33
S	27	+	33
	28	+	25
T	29	+	33
	30	+	29
U	31	+	28
	32	+	28
V	33	+	30
	34	+	28
Total.....10	18		Average.....30

* Right testicle inoculated.

† Rabbit 20 was an old animal with small testicles, and the reaction was of the atrophic type.

‡ Rabbit 23 was inoculated into both testicles with positive results in 33 and 39 days.

+ indicates infection.

One inguinal node was excised from seven rabbits, five of which had been inoculated 2 days previously with the usual tissue implant and two with a testicular emulsion injected into the scrotum. Salt solution emulsions of the nodes were made as in the previous experiments and injected into the right testicle of twelve normal rabbits as shown in Table IV. Although no spirochetes were found in

TABLE IV.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 2 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	No. of rabbit inoculated with lymph node emulsion.	Result of inoculation.*	Incubation period of orchitis.
			days
W	35	+	28
	36	+	30
X	37	+	28
Y	38†	+	38
	39†	+	36
Z	40	+	29
	41	+	29
AA	42	+	29
	43	+	29
BB‡	44	+	30
	45	+	33
CC‡	46	+	33
Total.....7	12		Average.....31

* Right testicle inoculated.

† Rabbits 38 and 39 were old animals with atrophic testicles which probably accounts in part at least for the longer incubation time of the orchitis.

‡ Rabbits BB and CC were inoculated by a scrotal injection of an orchitis emulsion instead of the usual tissue implant.

+ indicates infection.

any of the emulsions by dark-field examination, a marked syphilitic orchitis developed in all subinoculated rabbits with an average incubation time of 31 days. The inguinal glands which were removed from rabbits with tissue implants produced an orchitis which was detected 28 and 29 days after inoculation, with the

exception of two old animals with atrophic and less reactive testicles in which the orchitis developed more slowly with incubation periods of 36 and 38 days. The orchitis produced by the injection of the lymph nodes obtained from the two rabbits inoculated with a tissue emulsion was slightly longer, developing in 30 and 33 days, but obviously a much larger series would be necessary before any final comparisons could be made between the relative infectivity of the nodes resulting from the two types of scrotal inoculation.

The outstanding features of this experiment are first, the demonstration that only 48 hours after inoculation of the scrotum, the inguinal nodes contain active *Treponema pallidum*, and second, that the treponemata were constantly present in the inguinal nodes at this time. It is also worthy of comment that although no spirochetes could be demonstrated in the inguinal gland emulsions by dark-field examination, the orchitis resulting from the injection of the emulsions was of an actively developing type with an average incubation period of 31 days, and in half the cases of 28 and 29 days.

Time of Establishment of a Generalized Infection.

The previous experiments have shown that active treponemata capable of producing a rapidly progressive orchitis were constantly present in the inguinal lymphatic nodes of twenty-nine rabbits, 2 to 61 days after scrotal inoculation.

The question that next arose concerned the time of establishment of a generalized infection, since the inguinal glands are so rapidly infected after scrotal implantation although the development of the specific chancre reaction itself is usually not recognizable for some 10 to 14 days (3). In reality it is conceivable that many parts of the animal body might be infected shortly after local inoculation and that they in turn might serve as other foci for further dissemination of the virus. At any rate, we know that the inguinal nodes contain active treponemata as early as 2 days after scrotal inoculation, but on the other hand, such organisms might be chance invaders which had been rapidly disseminated from the primary focus of infection and, as such, would be incapable of independently sustaining an infection. Since there was no available information on these points, the following experiment was undertaken.

A series of ten rabbits was inoculated with tissue implants in the right scrotum only. 2 days later, under ether anesthesia, the entire right testicle with the scrotum was excised, leaving only a narrow circular strip of the posterior scrotal skin with which to close the wound. Healing took place promptly in each animal with no secondary infection. At the time of operation, no difference in the two inguinal lymph nodes could be made out by palpation and no specific reaction about the implant had occurred. The rabbits were kept under careful observation and the results of the experiments are shown in Table V. 40 days after operation, and 42 days after scrotal inoculation, a definite syphilitic lesion was

TABLE V.

Results Following Unilateral Amputation of the Right Scrotum and Castration of the Right Testicle, 2 Days after Inoculation of the Right Scrotum.

No. of rabbit.	Development of syphilitic lesions in left testicle.	Interval between inoculation and appearance of testicular lesions.	Presence of popliteal lymphadenitis.	Lesions of generalized syphilis, other than testicular.	Interval between inoculation and appearance of generalized lesions.
		<i>days</i>			<i>days</i>
A	+	42	+	Bone.	49
B	+	42	+	Skin.*	42
C	+	49†	+	Bone.	49
D	+	49†	+	"	45
E	+	49‡	+	" § skin.	45
F	+	42	+	"	49
G	+	42	+	"	75
H	+	49†	+	"	56
I	+	49‡	+	"	45
J	+	49‡	+	"	45

* Scrotal lesion.

† Suspicious orchitis—42 days.

‡ Orchitis of 3 or 4 days standing on the 49th day.

§ Lesion, right tarsus.

present in the left testicle or head of the epididymis in four rabbits and a suspicious lesion in three others. By the end of the 7th week after inoculation, the left testicle of all ten rabbits was the seat of a progressive nodular orchitis. In addition, definite periosteal nodules of the nasal bones in four cases and a skin granuloma on the outer side of the right tarsus in one case were also found at the time; *i.e.*, 45 days after inoculation. Bone lesions were found in four other rabbits 4 days later and in another animal by the end of the 8th week, while the last rabbit in the group showed definite bone lesions 75 days after inoculation. Other generalized manifestations consisting of cutaneous granulomata were observed in five rabbits during the 2 months succeeding the appearance of the first lesions.

The right inguinal lymph nodes were definitely enlarged and resistant for some 10 days after the operation and then became shot-like in character. Both popliteal nodes were likewise persistently enlarged and indurated in each rabbit of the series, this change being pronounced by the time of occurrence of the metastatic testicular lesions.

The consistent results of this experiment demonstrate that 2 days after scrotal inoculation an active syphilitic infection is sufficiently established in the rabbit so that it is no longer dependent upon the focus of primary inoculation as a source of spirochetes.

Localization of Spirochetes in Lymph Nodes Other than the Inguinals.

Other superficial lymph structures such as the popliteal, cervical, submental, auricular, axillary, and flank nodes may also become enlarged and indurated during the course of syphilitic infection of the rabbit following local inoculation, but with the exception of regional lymph nodes in the drainage area of an active lesion, the popliteals show the most conspicuous and persistent abnormalities, of which a resistance or induration is usually the most marked feature. In many instances, the popliteals may be the only nodes in which gross abnormalities are detected. In order to demonstrate the presence of *Treponema pallidum* in lymph nodes other than the inguinals which showed these clinical changes, a number of animal inoculation experiments were carried out, of which the following four examples are typical.

Rabbit A.—Inoculated in the right testicle with an inguinal lymph node emulsion. A syphilitic orchitis developed and spirochetes were demonstrated in fluid aspirated from the testicle 41 days after inoculation. Both popliteals were enlarged and resistant, and 51 days after inoculation, the left node was resected under ether anesthesia, emulsified with salt solution, and injected into the right testicle of two normal rabbits. At this time, there were no generalized syphilitic lesions and no spirochetes were seen in the lymph gland emulsion by dark-field examination. One of the subinoculated rabbits died from accidental causes, but in the surviving animal, a syphilitic orchitis, in which spirochetes were easily demonstrated, developed, with an incubation period of 43 days.

Rabbit B.—Bilateral scrotal inoculation. At the time of operation, 14½ months later, there were active skin granulomata on the outer side of the right hind foot, left ankle, and nose, and extensive residual ocular changes, not, however, active at this time. Both popliteals were extremely large and rather resistant with

some fluctuation. The right node was aspirated with a syringe and 2 cc. of opalescent fluid flowed into the syringe without suction. No motile spirochetes were seen by dark-field examination of this fluid, of which 0.5 cc. was injected into the testicles of two normal rabbits. A syphilitic orchitis subsequently developed in both rabbits with incubation periods of 39 and 42 days.

Rabbit C.—Bilateral testicular inoculation, with the popliteal lymph node of Rabbit B. At the time of operation, 3 months later, there was a nodular orchitis of 46 days duration and a keratitis in the right eye. Right popliteal lymph node slightly enlarged and indurated. Left popliteal lymph node, however, which had been enlarged and indurated for some weeks, was resected under ether anesthesia, emulsified with salt solution, and 0.5 cc. was injected into the testicles of two normal rabbits. No spirochetes were seen in the emulsion by dark-field examination. An orchitis, however, was detected in the first subinoculated rabbit in 22 days, with subsequent demonstration of spirochetes, and progressed in an active manner. The second subinoculated animal was discarded because of a subcutaneous abscess.

Rabbit D.—Bilateral scrotal inoculation. At the time of operation, 16 months later, there were cutaneous, subcutaneous, and periosteal nodules of the lower portion of the nose and a large cutaneous granuloma of the left ankle. A right axillary lymph node and one in the upper left flank had been enlarged and indurated for some months. Enlarged cervical lymph nodes consisting of several firm, fairly elastic nodules in the deeper tissues of the neck and one large node under the right maxilla near the midline had also been observed for a considerable period of time. The large submental node was aspirated with a syringe and 0.4 cc. of an opalescent fluid was withdrawn. No spirochetes could be demonstrated by dark-field examination. The fluid was injected into the right testicle of a normal rabbit, and a nodular syphilitic orchitis subsequently developed with an incubation period of 51 days.

These experiments demonstrate that during the course of the syphilitic infection of rabbits produced by local inoculation of testicle or scrotum, superficial lymph nodes are infected with *Treponema pallidum*. In Rabbit A, there were no generalized lesions, and in Rabbit C, although there was a keratitis, there were no lesions about the hind feet and legs at the time the popliteal node was excised, so that the presence of *Treponema pallidum* in these nodes cannot be ascribed to a drainage infection. In the case of Rabbit B, however, there were active syphilitic lesions of the lower extremities and about the nose, so that the factor of drainage must be included in the infection of the regional lymph nodes in this instance. Clinically, this added factor is usually expressed by the larger size and the tenseness of the

lymph nodes rather than by induration. In Rabbit B, an axillary and an upper flank node were also enlarged and indurated, a condition which was undoubtedly due to the presence of *Treponema pallidum*.

Duration of Lymph Node Infection.

The presence or absence of spirochetes in lymph glands during the various phases of syphilitic infection in the rabbit is of particular interest and importance and especially during the so called latent period in which no syphilitic lesions can be found. As we have pointed out in a previous paper (4), spontaneous recovery in the rabbit is frequently only temporary, and lesions of some kind may recur after the lapse of a few months and occasionally of more than a year. Such intervals are examples of true latency in which the rabbit is still infected, as evidenced by subsequent syphilitic lesions, but during which the animal organism is able, for the time being, both to restrain the progressive development of spirochetes and to protect itself from their harmful effects. During this latent period, there is but one condition to indicate that infection may still exist, and that is the presence of some degree of abnormality of the superficial lymph nodes, especially the popliteals. The changes noted in cases of latent infection vary from a shotty induration of small nodes to a moderate enlargement with some degree of induration. The method of animal inoculation was used to ascertain whether spirochetes were present in the lymph nodes of rabbits in which the infection was latent.

The eight rabbits used for this series of experiments had been locally inoculated 7 months to $4\frac{1}{4}$ years previously as is shown in Table VI. All had had characteristic local lesions, and the first six animals in the table had shown well marked generalized lesions. The eighth animal was a female whose inoculation on the vulva resulted in a typical chancre, but no succeeding generalized manifestations had developed. At the time of operation, six rabbits showed no lesions of any kind, while two showed slight lesions of an indifferent character in which no spirochetes could be demonstrated by dark-field examination, and it was subsequently shown that one of these was not syphilitic (5). The latent periods of infection in these animals varied from 3 to 25 months. All eight rabbits, however, showed a popliteal adenopathy varying from small shot-like glands which were atrophic and

fibrous to moderately enlarged and indurated glands which showed some degree of medullary swelling. The popliteal nodes were resected under ether anesthesia, emulsified with salt solution, and 0.5 cc. of the emulsion was injected into the right testicle of two normal animals.

The results of these experiments demonstrate the constant presence of *Treponema pallidum* in the popliteal nodes of rabbits during latent periods of a syphilitic infection. Moreover, the rapidity with which an orchitis developed in the subinoculated rabbits, with incubation periods of 31 to 44 days, is particularly striking. In both of these respects, the results practically coincide with those obtained from the inoculation of lymph gland emulsions from cases of acute or active syphilis, as shown in the preceding sections of this paper. The duration of infection or of the period of latency in the group of animals reported is not unusual, except in Rabbits A, G, and H. The infection in these animals had at all times been mild; only a few lesions developed and these were comparatively slight. Nevertheless, virulent organisms remained alive in Rabbits A and G for approximately $4\frac{1}{4}$ and 3 years. Rabbit A is by far the longest case of animal infection on record and indicates very conclusively that the apparent recovery which takes place in these animals is only a symptomatic one.

The subsequent history of the eight rabbits extends over many months. Five animals have shown no change whatever in their condition. In Rabbit D, a slight infiltration about the right nostril extended to the left and eventually presented the typical appearance of syphilitic lesions in this locality, while in Rabbit E, a keratitis developed later. In the case of Rabbit A, the lesion in the scrotum proved to be a malignant growth from which extensive metastases occurred to the right and left inguinal and left flank lymph nodes, lungs, liver, spleen, kidneys, and bone marrow, leading eventually to marked emaciation, asthenia, loss of sphincter control, and the development of trophic ulcers about the anus, so that the animal was killed.

It would seem, therefore, from the striking results of these experiments, that for long periods of time, *Treponema pallidum* may be present in the lymph nodes of rabbits which have recovered from clinical manifestations of the disease even though no further manifestations of the infection occur, and further that the reservoir of the organisms during latent periods of syphilitic infection is lymphoid tissue.

The Use of Lymph Node Reservoirs as a Means of Demonstrating Infection.

The data obtained from the various series of experiments described in this paper indicate the fundamental rôle played by lymphatic tissue in syphilitic infection of the rabbit. We know that dissemination of spirochetes from a local focus of inoculation in the scrotum to the inguinal lymph nodes occurs extremely rapidly and that during the course of an infection *Treponema pallidum* may be recovered from other superficial lymph nodes as well. This may be accomplished not only during active phases of the infection but in the so called latent period in which no manifestations of the disease are found except abnormal popliteal lymph nodes which contain treponemata of surprising infectivity.

Up to the present time, there has been no means at one's disposal to ascertain whether or not infection still persists in a rabbit if no local recurrence or generalized lesions develop, except to hold the animal for a prolonged period of time for such manifestations to appear. This has been the method employed in judging the results of the treatment of the experimental infection, but the use of lymph node inoculations offers an illuminating as well as a time-saving procedure for determining the ultimate or curative effect of a therapeutic agent, as illustrated by the following experiment.

Among a group of rabbits treated with arsphenamine and neoarsphenamine, there were five which showed no clinical recurrence during a period of 3 months observation. These animals had been inoculated in both testicles with the Nichols strain of *Treponema pallidum*; two rabbits were given single doses of 6 mg. per kilo of arsphenamine (German salvarsan Lot A 25819) and three rabbits 9 mg. per kilo of neoarsphenamine (German neosalvarsan, Lot A 25884). The drugs were administered intravenously in a 0.2 per cent solution; the arsphenamine was neutralized by the theoretical amount of N sodium hydroxide to form the disodium salt. The doses employed have been shown to produce definite therapeutic effects in the average animal, consisting of a regression of lesions approximating complete resolution with freedom from recurrence for 4 to 6 weeks followed by clinical relapse within a period of not more than 2 to 3 months (6).

The immediate therapeutic effects consisted in the regression and resolution of the testicular lesions, which proceeded somewhat more rapidly in those animals treated with neoarsphenamine, so that by

TABLE VI.

Demonstration of Treponema pallidum in the Popliteal Lymph Nodes during Latent Periods of Infection.

Source rabbit.	Duration of infection.	Length of latent period.	No. of rabbit inoculated with popliteal lymph node emulsion.	Result of inoculation.	Incubation period of orchitis.
	<i>mos.</i>	<i>mos.</i>			<i>days</i>
A*	51	6	1	+	38
			2	+	43
B	9	3	3	+	42
			4	+	44
C	7	3	5	+	37
			6	+	39
D*	9	3	7	+	34
			8	+	31
E	7	3	9	+	37
			10	Dead.	
F	7	3	11	+	31
			12	+	31
G	35	25	13	+	35
			14	+	35
H	10	7	15	+	34
			16	+	34

* These two animals showed suggestive lesions at the time of examination. They subsequently increased but no spirochetes could be demonstrated in them by dark-field examination. The lesion in the case of Rabbit A proved to be a malignant growth.

+ indicates infection.

the end of the 2nd week, the testicles were normal except for small residual areas of thickening or infiltration in four animals, which continued unchanged during the 3 months observation period following treatment.

Aspiration of the testicles for dark-field examination was not done because of the possible regressive effect of such a trauma. In addition no generalized manifestations of a specific character had developed. The only suggestive evidence of the existence of a still active infection 3 months after treatment was an enlargement and induration of the popliteal lymph nodes which were present in all five rabbits. In order to determine definitely the presence or absence of infection, the left

TABLE VII.

Demonstration of Treponema pallidum in the Popliteal Lymph Nodes 3 Months after Treatment with Arsphenamine and Neoarsphenamine.

Source rabbit.*	Interval between inoculation and treatment.	Treatment; amount per kilo.	Amount of popliteal lymph node emulsion injected.	No. of rabbit inoculated with popliteal lymph node emulsion.	Result of inoculation.	Incubation period of orchitis.
	days		cc.			days
A	18	6.0 mg. of arsphenamine.	0.3	1	+	37
			0.3	2	+	45
B	18	6.0 " " "	0.3	3	+	34
			0.3	4	+	34
C	18	9.0 mg. of neoarsphenamine.	0.4	5	+	34
			0.4	6	+	34
D	18	9.0 " " "	0.2	7	+	29
			0.2	8	+	31
E	18	9.0 " " "	0.4	9	+	29
			0.4	10	+	31

* All five rabbits presented a progressive nodular orchitis which was approximately at the height of its acute phase or cycle at the time of treatment.

+ indicates infection.

popliteal node was excised for inoculation into test animals. Upon removal, the nodes showed a slight degree of enlargement, slight fibrosis of the surrounding tissue, and slight thickening of the capsule; they were translucent and firm with prominent lobulation. A salt solution emulsion of each node was prepared and from 0.2 to 0.4 cc. was injected into the right testicle of two normal rabbits as shown in Table VII.

The noteworthy features of this series of experiments are first, the constancy with which positive results were obtained in each testicular subinoculation, showing that *Treponema pallidum* was present in the popliteal lymph nodes of each rabbit, and second, the infectivity of the treponemata as shown by the rapidity with which the orchitis developed. In eight instances, a testicular lesion was recognized in 29 to 34 days with an average incubation period of 32 days, while in two rabbits, the orchitis developed somewhat more slowly—37 and 45 days. These results are strikingly similar to those obtained from test inoculations of popliteal lymph nodes of latent cases of syphilitic orchitis and are in close approximation to those observed from the inoculation of inguinal lymph nodes of cases with active scrotal lesions.

It might be briefly stated that the three rabbits treated with neoarsphenamine and one of the two treated with arsphenamine subsequently showed an outspoken clinical relapse but that no characteristic lesions developed in the other during a 4 months period of observation following treatment.

The possibility of the recovery of spirochetes from lymph node reservoirs is further emphasized by the results obtained from the continued passage of *Treponema pallidum* from lymph node to testicle. For about 18 months we have carried the Nichols strain of *Treponema pallidum* in eight serial inoculations of inguinal or popliteal lymph nodes to testicles of normal rabbits, thus demonstrating the ability to recover spirochetes from lymphatic tissue through successive generations. With such a procedure, the orchitis tends toward a more prolonged or chronic type with a distinctly longer incubation period which averaged from 4 to 6 weeks. Not infrequently, the reaction consisted in a primary atrophy of the testicle rather than an enlarged granulomatous change. That such a condition is one type of primary response toward *Treponema pallidum* is shown by dark-field demonstration of spirochetes. Moreover, in doubtful cases when no lesions of the testicle can be detected by palpation, infection may be recognized by an adenopathy of the superficial lymph nodes.

Finally in this connection, reference should be made to the use of lymph node reservoirs for preserving stock strains of *Treponema pallidum* when not in active use, thus obviating the necessity of frequent testicular or cutaneous transfers (7).

DISCUSSION.

The studies reported in the present paper were undertaken for the purpose of ascertaining the relation of *Treponema pallidum* to lymphoid tissues in syphilis of the rabbit produced by local inoculation. The problem was approached by a series of experiments designed to show the frequency, time, and extent of lymphatic dissemination of spirochetes from a primary focus of infection in the scrotum or testicle, and the method employed consisted of the demonstration of *Treponema pallidum* in the lymph nodes of infected rabbits by animal inoculation.

The first series of experiments was carried out with emulsions of satellite nodes from rabbits which had been inoculated in the scrotum 2 to 61 days previously. Nodes from twenty-six animals produced a syphilitic orchitis when injected into the testicles of normal rabbits, and spirochetes were found in three others, thus demonstrating that a dissemination of organisms from a primary focus of inoculation constantly occurs by way of the lymphatic system. In only one out of forty-six rabbits inoculated did a progressive orchitis fail to develop, and in this instance, the orchitis was of the atrophic type. In contrast to the high infectivity of the node emulsions is the small number of spirochetes that must have been present in the nodes, since organisms were demonstrated by dark-field examination in only three out of twenty-nine emulsions.

The lymph node emulsions contained little or no microscopic blood, and, in addition, parallel experiments have shown that inoculations of as much as 0.5 cc. of blood rarely produce an infection with an incubation period of 5 weeks.

The second point of importance emphasized by the results of these experiments is in regard to the time of dissemination of the spirochetes from the local focus of inoculation in the scrotum. At the time this work was done, the inguinal lymph nodes showed definite changes, consisting of an enlargement and tenseness or resistance within 4 or 5 days after scrotal inoculation and ultimately progressed to a condition of marked enlargement and induration. With the knowledge that has been gained of the constant dissemination of spirochetes from the scrotum, at least as far as the satellite nodes are concerned, one might logically assume that the early changes in the nodes

signified the presence of spirochetes. That this assumption is justified, has been borne out by the consistently positive results of the series of rabbits in which the inguinal nodes were removed 5 and 7 days after scrotal inoculation. The striking feature in regard to the time of dissemination, however, is revealed by the constant infectivity of the inguinal lymph nodes only 2 days after scrotal inoculation, at a time when no gross abnormalities were detected, and, in addition, when no syphilitic reaction about the chancre implant could be recognized. Moreover, it seems justifiable to assume from the evidence at our disposal that enlargement and tenseness of the inguinal lymph node occurring at present as early as 24 hours after scrotal or sheath inoculation are due to the presence of *Treponema pallidum*.

The character or degree of the infection present in the nodes as indicated by the type of orchitis produced by the inoculation of the node emulsions is of considerable interest. There were 57 inoculations made, four of which are omitted from the present analysis (accidental death, etc.). The average incubation period of the orchitis which occurred in the remaining 53 animals determined by the development of palpable lesions was 31 days; in twenty-five animals, it was 29 days or less, and the longest incubation period was 41 days (two animals). Examination of testicular fluid would doubtless have shown the presence of an infection much earlier, but was not carried out on account of the traumatism which may obscure subsequent changes.

It has further been shown that within 48 hours after scrotal inoculation spirochetes have become disseminated and sufficiently established in the animal body to maintain an active progressive infection in the event of the removal of the scrotum with the implant and testicle. Syphilitic lesions of the remaining testicle and epididymis developed in each of the ten rabbits comprising the series in from 40 to 46 days after operation, and, in addition, well marked generalized lesions in each animal were a striking feature of the experiment. These results are not to be interpreted as implying that the inguinal lymph nodes constitute the only focus of infection under the conditions of the experiment, but since it has been shown that spirochetes are constantly present in the inguinal nodes 2 days after scrotal

inoculation, it may be assumed that they serve as one such focus. In any event, this experiment demonstrates that the infection itself is so established within 2 days after scrotal inoculation that it is independent of the primary lesion as a source of spirochetes.

The general tendency of *Treponema pallidum* to localize in lymphatic tissue is also shown by the recovery of organisms from other nodes, such as the popliteal and submental. It further appears that such localization is not dependent upon an active lesion in the drainage area of the node but that a widespread dissemination of spirochetes from the local focus of infection occurs by way of the lymphatics. Thus, enlargement and induration of various lymph glands which may occur during the course of a syphilitic infection are significant of the presence of *Treponema pallidum*, and since a popliteal adenitis is a constant feature of syphilis in the rabbit, this condition may be considered as pathognomonic of infection.

The persistence of active spirochetes in the animal body for long periods of time without manifestations other than some abnormality of the popliteal nodes is a fundamental concept of syphilitic infection in the rabbit. We have shown that in cases of true latency of from 3 to 25 months duration, spirochetes may be recovered from the popliteal nodes by animal inoculation. The noteworthy features of this series of experiments were the constancy with which the nodes were shown to harbor active treponemata and the rapidity with which the resulting orchitis developed, the incubation period varying from 31 to 44 days. These results, moreover, are practically the same as those obtained by the testicular inoculation of inguinal lymph nodes from early or active cases of infection.

It would appear, therefore, that although the rabbit possesses some type of mechanism during latent periods of the infection which operates to protect the animal from the harmful toxic effects of the organisms and prevents the development of manifestations of the disease, the organisms themselves are not destroyed but are existent in lymphatic tissue in an active state. Whether other tissues may serve as similar foci for the maintenance of active treponemata for long periods of time is not known, but the well known affinity exhibited by *Treponema pallidum* for lymphatic tissue, together with the clinical evidence of continued adenopathy in latent cases of infection, would

point toward this tissue and especially its larger aggregations—the lymphatic nodes—as the principal reservoirs of the virus.

A close similarity existed between the orchitis produced by lymph node inoculation and that caused by an emulsion of either an infected testicle or a skin nodule such as is frequently employed in propagating *Treponema pallidum*. The incubation period of the orchitis produced by inoculation of a node emulsion was about 7 to 10 days longer, but once the process had reached the stage when it could be diagnosed by palpation, its further development proceeded in much the same manner and at about the same speed. This is the more remarkable when the spirochete content of the two inoculating emulsions is considered, spirochetes being numerous in emulsions prepared from a testicular or skin nodule, while it is unusual to see a single organism in those from a lymph node. Thus we are forced to the conclusion by the results of these experiments that actual numbers of spirochetes may play a very small rôle as a causative factor in producing an active progressive syphilitic orchitis and that the state of vitality or degree of infectivity of the spirochetes is of much greater importance.

The ability to recover spirochetes from lymphatic tissue is further emphasized by the results of the serial passage of lymph node emulsion to testicle. During the last 18 months, there have been eight transfers of this type, and in every instance it has been possible to produce a syphilitic orchitis by the inoculation of a lymph node emulsion, the infection of which had in turn resulted from a testicular injection of a lymph node emulsion.

The facts derived from this group of experiments may be profitably utilized in many ways. We have suggested a simple method based upon the duration of lymph node infection for the preservation of stock strains of *Treponema pallidum*, which is both economical and time-saving, and, in addition, is especially effective in guarding against loss of the strain.

A further utilization of the facts derived from these experiments lies in the demonstration of infection in rabbits which show no manifestations of disease as a result of the administration of therapeutic agents. The methods commonly employed in judging the results of therapy have consisted in observations on the effect of the agent upon the spirochetes and upon the regression, healing, and recurrence of

the local lesion during observation periods of from 1 to 3 months. In our experience, however, we have seen many instances of a relapse in regions remote from the original lesion with no local recurrence, and, in addition, we know that either a local or a generalized relapse may occur considerably longer after treatment than 3 months. Such rabbits may show a characteristic popliteal adenitis, or there may be no distinctive adenitis, but with the knowledge which has been gained of the persistence of *Treponema pallidum* in lymph node structures, it is possible to demonstrate the presence of spirochetes by animal inoculation without waiting for a clinical relapse. This was done in the case of five rabbits treated with arsphenamine and neoarsphenamine which had shown no local or general manifestations of disease during the 3 months observation period following treatment. The popliteal nodes excised from these rabbits were inoculated into the testicles of normal rabbits with the production of a syphilitic orchitis in all instances. Subsequently four of the five rabbits showed typical local recurrences.

SUMMARY.

A widespread dissemination of *Treponema pallidum* from a local focus of inoculation in the rabbit constantly occurs by way of the lymphatics. Spirochetes were regularly recovered from the satellite lymph nodes by animal inoculation after scrotal inoculation; they were present as early as 2 days, when no specific primary reaction was detected, and at later periods of from 5 to 61 days after inoculation. Other superficial nodes at remote sites such as the popliteals and with no syphilitic lesions in the drainage area have also been shown to harbor active organisms. Although spirochetes were found in relatively few of the lymph node emulsions, the orchitis resulting from their injection was of a rapidly progressive type with an incubation period but slightly longer than that produced by a testicular or skin nodule emulsion rich in spirochetes.

It has further been shown that a syphilitic infection is sufficiently established in the rabbit body within 48 hours after scrotal inoculation so that the primary lesion is no longer essential for its maintenance.

Active treponemata survive in the popliteal lymph nodes for long periods of time and have been regularly recovered from them in cases

of true latency. The lymph nodes, therefore, function as reservoirs of the organisms. The ability to recover the spirochetes from lymphoid tissue through successive generations is seen in the serial passage of lymph node emulsion to testicle during an 18 months period.

The persistence of spirochetes in lymphoid tissue irrespective of the presence or absence of syphilitic lesions is a characteristic and fundamental feature of syphilis of the rabbit. The existence of infection, therefore, may be demonstrated at any time by the recovery of spirochetes from the popliteal lymph nodes by animal inoculation. This fact is of great practical importance in the therapy of the infection and may be profitably utilized in determining the ultimate effect of a therapeutic agent.

These experiments demonstrate that the disease is not confined to the site of local inoculation but that lymphogenous dissemination of treponemata regularly takes place, and that during the course of this process organisms become localized in the lymph nodes and exist there indefinitely irrespective of the occurrence of manifestations of disease. The intimate relation of *Treponema pallidum* to lymphoid tissue is an essential concept of syphilis of the rabbit, and from this point of view, the infection is primarily one of lymphoid tissue.

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STUDIES ON AGGLUTINATION WITH THE AID OF THE CENTRIFUGE. THE INFLUENCE OF TEMPERATURE ON ABSORPTION AND FLOCCULATION.

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Whatever opinions may be held regarding the nature of the specific agglutination of bacteria it is generally agreed that the process occurs in two phases; a reaction between cells and serum, in which the antigen and the specific antibodies combine, and a secondary reaction among the affected bacteria in which they cohere and flocculate out of suspension.¹

Both phases of agglutination proceed gradually to completion and are subject to variation according to the physical conditions under which they occur. Thus, temperature, agitation, mass action, and the influence of electrolytes are known to affect the phenomenon. In the usual agglutination technique the spontaneous flocculation of the bacteria serves as the index of the primary reaction between cells and serum. The complete process, however, includes all the variable factors to which the flocculation phase is subject, and thus the conditions under which the absorption phase proceeds are not open to separate analysis. A method of examination which would eliminate the unknown time element in the flocculation phase, and thus afford a cross-section view of the status of the absorption phase at any period in its progress, might be of value in a closer analysis of some of the factors affecting absorption and perhaps throw some light on the nature of the reaction. The flocculation of bacteria in the presence of their specific antisera may be mechanically effected by means of the centrifuge. The affected organisms, brought into contact by centrifugation, tend to adhere in clumps identical with those produced by the unaided process of agglutination.

¹ For the purposes of this paper the current terms absorption and flocculation have been accepted for these phases of the reaction.

Gaehstgens² (1906) advocated the use of the centrifuge in agglutination tests with *B. typhosus*. Small, round bottomed tubes of serum and bacterial suspension were centrifuged for 10 minutes without previous incubation, and the resulting sediment was examined macroscopically and microscopically for evidence of clumping. In positive instances a characteristic sediment was formed, which broke up into the typical flocculi of agglutination on shaking, and on microscopic examination showed large clumps of adherent bacilli. The results of centrifuge agglutination with the sera of 100 persons duplicated the findings by the usual method. Control sera, producing no agglutination in the incubator, gave uniformly negative results with the centrifuge. In 1907 and 1908, Brian³ and Gaehstgens⁴ applied the centrifuge method to meningococcus agglutination, which requires 16 to 24 hours incubation at 55°C. by the standard method.

In spite of obvious advantages in point of time and of more exact control over the conditions of test, Gaehstgens' method seems to have attracted little attention. Its failure to find a place among bacteriologic methods may have been due in part to the technical disadvantage of centrifuging the large numbers of tubes often used in agglutination tests, and in part to the confusion which attended the serological diagnosis of the meningococcus—a field in which it might have been useful—before the variations in agglutinin response had been explained by Dopter. Gaehstgens' method seems to have survived for a time only in the diagnosis of glanders, and to have fallen into disuse.

During the period of the war we employed the centrifuge method of agglutination in an extensive series of diagnostic and analytical experiments with the meningococcus group of organisms. In many parallel tests the method was found to be entirely reliable, giving results closely in accord with those obtained by the standard method of incubation at 55°C. for 16 hours.

In the course of these experiments a number of observations were made bearing on certain physical conditions which affect the agglutination reaction. Most of these observations were made on the behavior of meningococci in their specific homologous antisera. These organisms proved to be well adapted for such studies because of the slow rate at which agglutination ordinarily proceeds. The experiments which dealt primarily with the effect of temperature on absorption and flocculation have been collected in this report.

² Gaehstgens, W., *Münch. med. Woch.*, 1906, liii, 1351.

³ Brian, O., *Centr. Bakt., 1te Abt., Orig.*, 1907, xliii, 745.

⁴ Gaehstgens, W., *Arch. Hyg.*, 1908, lxvi, 377.

The Centrifuge Method of Agglutination.

A standardized technique was maintained throughout except as noted in single experiments. The essentials of the method are as follows:

The meningococcus suspensions used were heat-killed cultures which were found to behave in a uniform manner over a period of months.⁵ The organisms were grown on glucose agar in Blake bottles for 16 hours at 37°C., washed off with 10 to 20 cc. of sterile 0.8 per cent salt solution, sealed in small bottles, and immediately heated to 65°C. for 30 minutes in a water bath. These concentrated stock suspensions, containing 20 to 50 billion organisms per cc., were stored at 4°C. without a preservative. In some instances the straw-colored supernatant fluid was replaced with sterile saline solution. Dilutions of 2 to 4 billion cocci were made and standardized⁶ as required.

Immune serum of rabbits or horses was also stored at 4°C. without a preservative and the successive dilutions were made up fresh for each experiment.

Usually 1 billion organisms were suspended in 1 cc. of dilute serum. The tests were carried out in round bottomed test-tubes, 9 by 90 mm., without lips. They were incubated in a water bath or thermostat, as required by the conditions of the experiment, and centrifuged in groups of five to seven in the wide cups used in the Babcock milk test. An eight cup head on the electric centrifuge thus accommodated 40 to 56 tubes at one time. The specimens were uniformly centrifuged at a speed just sufficient to deposit unagglutinated organisms—10 minutes at 1,800 revolutions per minute.

After centrifugation the tubes were agitated to bring the sediment into suspension and the results read macroscopically by oblique transmitted light against a dark background. Examined in this manner the evidences of agglutination are unmistakable. Unagglutinated organisms, in normal serum or in salt solution, on centrifugation form a compact button in the bowl of the tube. On shaking slightly the sediment comes up in a smooth corkscrew whirl and is evenly distributed throughout the liquid. In the presence of an active serum, however, the central button is surrounded by a fringe of feathery flocculi spread over the bottom of the tube. This flaky layer was well described by Gaechtgens.² On shaking, the sediment rises in small clumps or larger masses in a clear or turbid fluid, depending upon the degree of agglutination present. The appearance is identical with that found after spontaneous flocculation. In our experiments three degrees of agglutination were recognized: complete, all the organisms compactly clumped in a clear liquid; incomplete, abundant clumps in a slightly hazy liquid; and partial, small but distinct clumps in a cloudy liquid. In the

⁵ Amoss, H. L., Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1920, xxxii, 778.

⁶ Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

charts these states are indicated by black bands of different widths, the broad bands indicating complete agglutination. The experiments were carried out with the usual controls in normal serum and salt solution.

Comparison of Agglutination of Meningococci by the Standard and the Centrifuge Methods.

When Brian and Gaehtgens published their observations nothing was known of the specific variations among meningococci which led to the establishment of types by Dopter and their confirmation by Gordon and by Nicolle, Debains, and Jouan. The first problem, therefore, in the application of the centrifuge method to immunological studies with meningococci was to examine the method for type specificity in comparative tests with the standard agglutination technique.

This has been done repeatedly in many experiments with meningococci of spinal or nasal origin, in normal sera and in monovalent or polyvalent antisera from the rabbit or the horse. Typical examples of such comparative tests are shown in Text-fig. 1, for the two main types of meningococci, Types A and B,⁷ and for strains which on agglutination show an intermediate character, coming down in almost equal dilutions of the antisera of each type.

Experiment 1.—Type A serum, Horse 33. Type B serum, Horse 32. Sera 1 month old. Serum dilutions 1:50 to 1:400. 1 billion heat-killed organisms suspended in 1 cc. of dilute serum. Duplicate sets. One set centrifuged immediately, shaken, and read. The other set incubated 16 hours at 55°C., shaken, and read. Results in Text-fig. 1.

In this experiment it may be seen that centrifugation without preliminary incubation gives somewhat more clear-cut results in Groups A and B, while in the group of intermediate strains the results by both methods are practically identical. The specific immunological differences among meningococci are thus shown to be discernible by this method, and indeed are accentuated rather than decreased, so that the centrifuge method is, perhaps, the more useful

⁷ Following Dopter we accept the classification of Nicolle, Debains, and Jouan, in which the meningococcus is designated Type A, and Dopter's parameningococci, alpha, beta, and gamma, are called Types B, C, and D respectively.

for type diagnosis. These differences are probably due in part to the fact that the so called coagglutinins seem to react more slowly than do the specific agglutinins and require a longer incubation time for their demonstration. A discussion of this interesting difference is not within the scope of this paper.

Serum type	Centrifuge method				Standard method			
	A		B		A		B	
Dilutions	1:400	1:200	1:100	1:50	1:400	1:200	1:100	1:50
No. 2								
31								
Type A strains								
36								
60								
65								
No. 1								
6								
Type B strains								
8								
71								
84								
Inter- No. 5								
-mediate 30								
strains 32								
61								
66								

TEXT-FIG. 1. Centrifuge agglutination of meningococci without incubation *versus* the standard method, incubated 16 hours at 55°C.

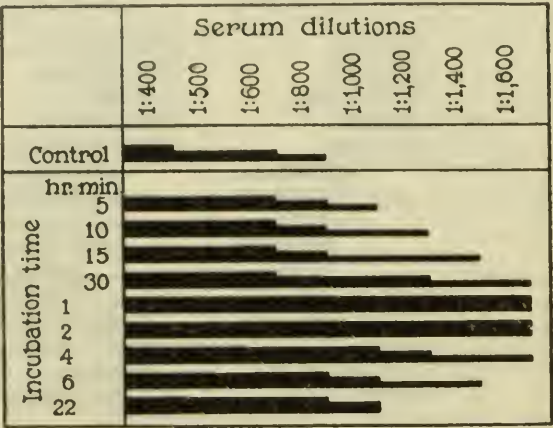
The Effect of Incubation at 55°C. on the Serum Titer.

In general, centrifuge agglutination without incubation has given results of somewhat lower titer than has spontaneous agglutination after 16 hours incubation at 55°C. This fact led us to combine a short incubation period with the mechanical flocculation, and in this way the highest titers with a given serum were obtained. These observations were based on experiments such as the following.

Experiment 2.—Type B meningococcus, Strain 1, agglutinated in homologous monovalent antiserum from a rabbit. Suspensions of the killed culture, in successive dilutions of the antiserum, were centrifuged without incubation to determine the approximate limits of agglutination. Beginning with serum dilutions near the upper limit of complete agglutination, coccus-antiserum mixtures were set up at close dilution intervals, incubated at 55°C. for periods of 5 minutes to 22 hours, centrifuged, shaken, and read. The results are given in Text-fig. 2.

A period of incubation promotes absorption to such an extent that complete clumping occurs in dilutions two to four times higher than

without previous incubation. Even 5 minutes exposure to 55°C. has an obvious effect. The highest serum titer is found after 1 to 4 hours incubation. Long exposure, 16 to 24 hours, to 55°C. may cause a diminution in the titer. This observation has been made repeatedly and is due to a deleterious effect of the high temperature on the serum antibodies, as will be shown presently. Repetitions of Experiment 2 with other strains and sera have given practically identical results.



TEXT-FIG. 2. The effect of incubation at 55°C. on the titer of agglutination in antimeningococcus serum.

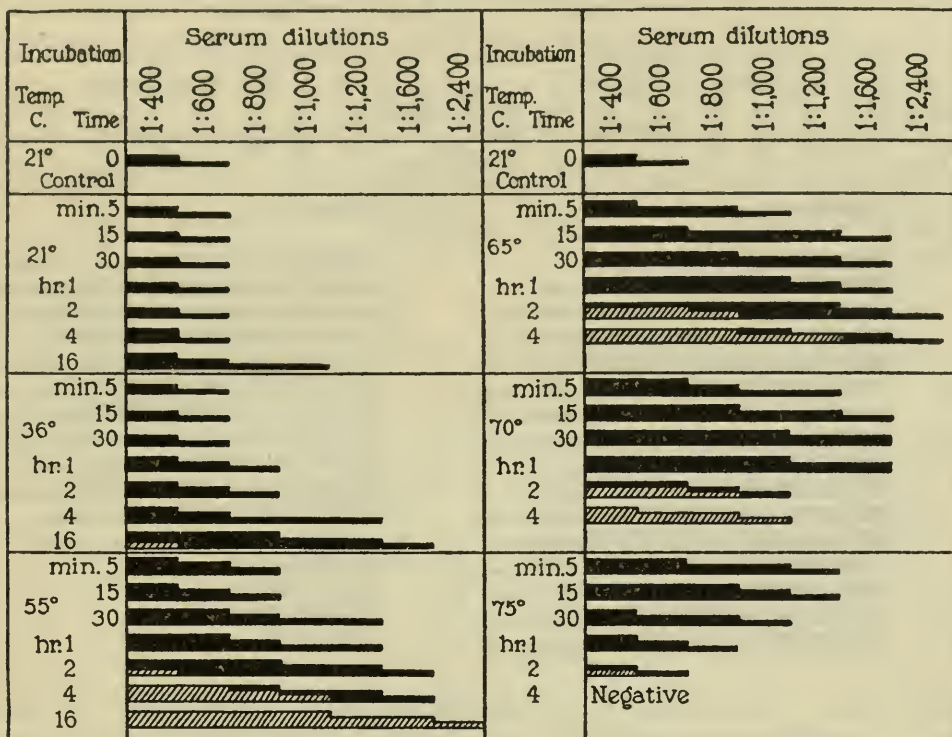
The Effect of Temperature on Absorption.

The foregoing experiment shows that contact of organisms and antisera for 1 to 4 hours promotes a reaction sufficient to produce clumping on centrifugation in high dilutions of the antiserum. Is the temperature at which the reaction takes place a function of the time required for its completion?

Experiment 3.—Suspensions of a Type B organism, Strain 1, were incubated at various temperatures and for varying periods of time in successive dilutions of a homologous monovalent antiserum from a rabbit. After the determined incubation period (water baths) the tubes were read to note the occurrence of spontaneous flocculation, and were then centrifuged, shaken, and read. The results are given in Text-fig. 3.

In the preliminary test, without incubation, complete agglutination occurred at 1:200 (not shown on the chart) with the limit of visible clumping at 1:600. In these serum dilutions the bacteria

absorbed enough agglutinin during their passage to the bottom of the tube to cause them to cohere in spite of resuspension. This reaction is a constant in each experiment and forms the base-line in each test. No more absorption than this occurred at 21°C. after 4 hours contact, but after 16 hours some agglutination was found in a dilution of 1:1,000. At 36° absorption proceeded more rapidly and a positive reaction was evident after a contact of 1 hour. As already noted

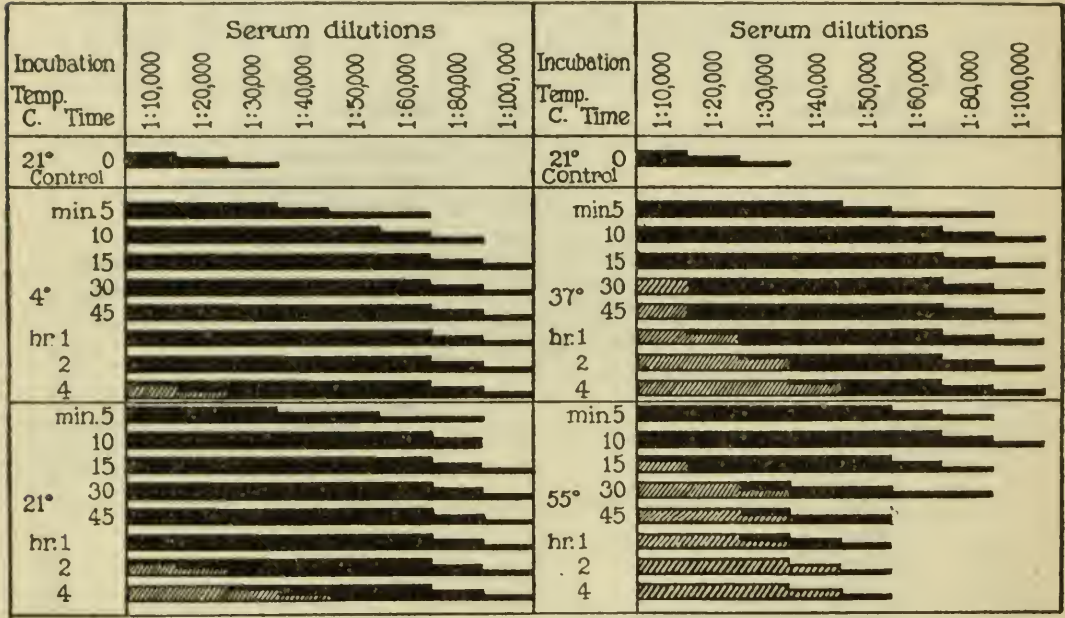


TEXT-FIG. 3. The influence of temperature on the absorption of meningococcus agglutinins. Spontaneous flocculation cross-hatched; centrifuge agglutination in black.

in Experiment 2, 5 minutes contact at 55° promotes absorption, which, however, did not reach the limit, 1:2,400, until more than 4 hours had elapsed. At the higher temperatures the effect of heat is more quickly apparent, so that at 65° the limit of the reaction was reached in 2 hours and at 70° and 75° the maximum absorption occurred at 30 and 5 minutes respectively. At these high temperatures the serum antibodies were already in process of destruction so that the titer fell off rapidly as incubation was prolonged. Similar

results were obtained in a parallel experiment with a Type A meningococcus in its antiserum.

Although the agglutination reaction is not well adapted to accurate quantitative estimation because the end-point is not sharp, it is interesting to note in these experiments that the time and temperature relations are roughly those of chemical acceleration. The behavior of the serum is also similar to that of enzymes, which are most active just below the temperature of rapid destruction.



TEXT-FIG. 4. The influence of temperature on the absorption of typhoid bacillus agglutinins. Spontaneous flocculation cross-hatched; centrifuge agglutination in black.

It is only with an organism such as the meningococcus which reacts with its antiserum at a measurable rate that such an experiment can readily be performed. *Bacillus typhosus* reacts with its specific antiserum so rapidly that the process seems to be complete in 15 minutes at 4°C. and in 10 minutes at 36°C.

Experiment 4.—Suspensions of heat-killed typhoid bacilli in high dilutions of antiserum from a horse. Identical sets of tubes incubated at various temperatures for varying periods of time. Read to note spontaneous flocculation, centrifuged, shaken, and read. The results are given in Text-fig. 4.

High temperature quickly showed an injurious effect on the *Bacillus typhosus* agglutinins. The titer of the serum began to fall off after an exposure of 10 minutes at 55°C.

The Effect of Temperature on Flocculation.

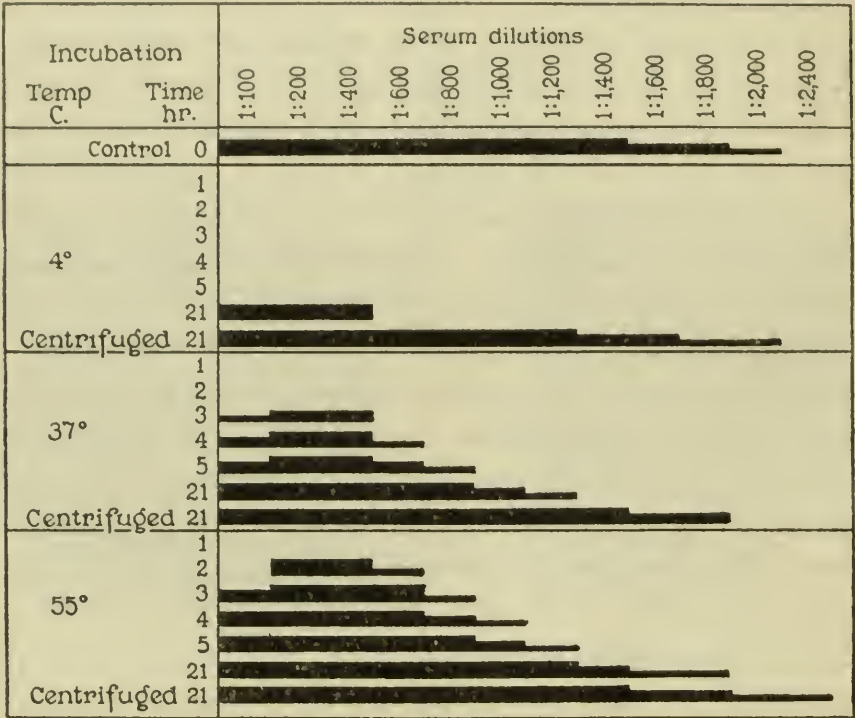
From the readings of spontaneous flocculation obtained in Experiments 3 and 4 before centrifugation of the specimens it was found that the higher temperatures accelerate the flocculation phase of the agglutination reaction also. These readings are indicated in Text-figs. 3 and 4 by cross-hatched areas within the solid black bands.

Although the centrifugation of the incubated specimens of Experiment 3 shows that some absorption had occurred in 16 hours at 21°C. and even in 1 hour at 36°, practically no unaided flocculation resulted at these temperatures in the serum dilutions at which the tests were made. At higher temperatures flocculation appeared earlier and in higher dilutions, but in no instance did it become visible in less than 2 hours, even though the absorption phase had reached its limit in 15 to 30 minutes. Most of the incubation time involved in spontaneous agglutination is required by the relatively slow development of visible flocculation. The influence of temperature upon flocculation is more strikingly shown in the experiment with *Bacillus typhosus*. Here absorption was complete in 15 minutes at 4°C. At this temperature flocculation was not evident for over 2 hours, whereas at 37°C. it appeared in 30 minutes in the lowest dilution examined, and at 55° it appeared in 15 minutes.

In the following experiment with a Type B meningococcus and its homologous antiserum absorption was completed in a short time by exposure to a high temperature and the subsequent flocculation allowed to proceed at various temperatures to determine the relative rate of its development.

Experiment 5.—Type B meningococcus, Strain 1. Homologous monovalent antiserum from a rabbit. Four sets of killed culture suspensions in successive serum dilutions. Heated at 70°C. for 30 minutes. One set centrifuged immediately as a control to determine the completion of the absorption phase. Other sets placed at 4°, 37°, and 55°, and examined at intervals. After 21 hours all the sets were centrifuged to confirm the completion of absorption. The results are given in Text-fig. 5.

These observations on the time and temperature relationships of the flocculation phase reveal the handicap that attends an attempt to study the specific phase of the agglutination reaction with spontaneous flocculation as the indicator. An analysis of these experiments shows that even at high temperatures flocculation does not become visible until long after absorption has progressed to the point required for complete agglutination, while at low temperatures, in high dilutions of the antiserum, flocculation may not appear at



TEXT-FIG. 5. The influence of temperature on meningococcus flocculation.

all, even though absorption has advanced appreciably. In order to produce a visible result, spontaneous flocculation requires an absorption reaction several times in excess of that required by the centrifuge method of agglutination.

The Destructive Effect of High Temperatures on Meningococcus Agglutinins.

The decline in agglutination titer which results from prolonged incubation at high temperatures is illustrated in the foregoing

experiments. Such a destructive effect was extensively studied by Eisenberg and Volk (1902), by Joos (1903), by Kraus and Joachim (1904), and by Scheller (1904-05). The following experiment, showing that this deleterious effect is exerted on the serum antibodies rather than on the bacteria, is cited to show the application of the centrifuge method to problems of this character.

Experiment 6.—Year old polyvalent antimeningococcus serum, in successive dilutions, and killed culture suspensions of a number of meningococcus strains, 2 billion per cc., were each divided into three lots and one lot of each was heated at 70°C. for 30 minutes, an incubation period found to have an injurious effect in agglutination tests with these materials. Tests were then set up with mixtures of (a) unheated serum and unheated organisms (control); (b) unheated serum and

Serum dilutions	Control					Culture heated					Serum heated				
	1:100	1:200	1:400	1:800	1:1,600	1:100	1:200	1:400	1:800	1:1,600	1:100	1:200	1:400	1:800	1:1,600
Strain No.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
63	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
66	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
76	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

TEXT-FIG. 6. The destructive effect of high temperatures on agglutinins.

heated organisms; and (c) heated serum and unheated organisms. These tests were incubated at 55°C. for 4 hours, centrifuged, shaken, and read, with the results shown in Text-fig. 6.

It is seen that the unheated serum and heated cocci react in a manner similar to the controls, whereas the heated serum has lost much of its agglutinating activity. Experiments already cited show that the antigen-antibody complex, after the specific reaction has occurred, is still subject to injury by high temperatures. In Experiment 3, for example, the decline in the agglutination limit on prolonged exposure to 65°, 70°, or 75°C. occurred after the absorption reaction was complete at these temperatures. Tests with antisera which have been stored for some time show that they are particularly

subject to the effects of high temperatures. The typhoid serum used in Experiment 4 was 3 years old, which may account for the decline in the agglutinin titer even at 55°C.

The Relation of Temperature to Mass Action.

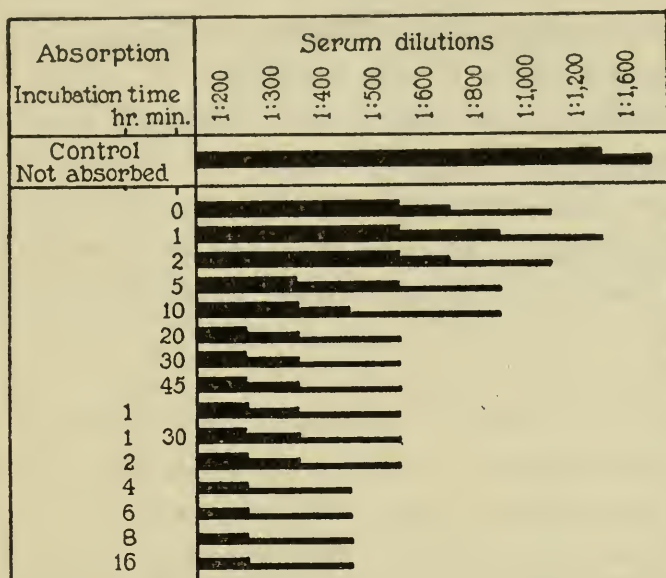
A survey of Text-fig. 3 shows that 0.001 cc. of the antiserum in a volume of 1 cc., a dilution of 1:1,000, contained enough agglutinins completely to clump 1 billion homologous organisms. This complete reaction required more than 4 hours for its development at 55°, but developed in 1 hour at 65° and in 30 minutes at 75°. In lower dilutions, containing an excess of antibodies, a reaction sufficient to clump all the organisms occurred much earlier at these temperatures, and even at 36° and 21° it occurred in the 1:400 dilution in 1 and 4 hours respectively. The presence of an excess of agglutinins greatly accelerates absorption. This fact has long been known, as has the fact that a given bacterial suspension may remove from an excess of serum several times the amount of agglutinins required for its complete flocculation (Eisenberg and Volk⁸). It is interesting to note, however, with the aid of the centrifuge, how quickly the process is brought to its conclusion.

Experiment 7.—Tubes containing 4 billion Type A meningococci, Strain 60, in 2 cc. of homologous antiserum diluted 1:100, were incubated at 55°C. for various periods of time. After centrifugation the clear, supernatant fluids were decanted and diluted successively, mixed with fresh lots of cocci (1 billion organisms in 1 cc. of serum), incubated at 55° for 4 hours, centrifuged, shaken, and read. The results are given in Text-fig. 7.

From the results of the control agglutination it is seen that the serum in a dilution of 1:1,200 originally contained enough antibodies completely to agglutinate 1 billion meningococci, a quantity which may be designated as 1 unit of agglutinin. Before absorption, therefore, each 2 cc. specimen, 1:100, contained 24 agglutinin units. The mere act of centrifugation, without incubation, in contact with 4 billion organisms, deprived the serum of about 12 units of agglutinin, leaving it with a titer of 1:500 to 1:600, so that each billion meningococci removed about 3 agglutinin units. 1 or 2 minutes

⁸ Eisenberg, P., and Volk, R., *Z. Hyg. u. Infektionskrankh.*, 1902, xl, 155.

incubation did not increase absorption appreciably, but during 5 minutes contact at 55° the serum titer dropped to 1:300, a loss of 18 units, and during 20 minutes at 55° the titer fell to 1:200, indicating the absorption of about 20 units. Each billion meningococci had absorbed about five times the agglutinins necessary for their complete flocculation. This appears to have been about the absorption limit, at this serum dilution, for after 20 minutes only a few more agglutinins were removed from the serum. In other experiments the reaction came to a standstill after 30 and 90 minutes respectively. It is evident that the incubation of absorption tests



TEXT-FIG. 7. Agglutinins left in antimeningococcus serum after absorption at 55°C. with the homologous organisms for various periods of time.

for long periods of time does not result in the removal of correspondingly large quantities of agglutinin. Repeated absorption with fresh bacteria over short incubation periods is the more effective method of removing specific antibodies from serum.

SUMMARY.

The flocculation of bacteria which have absorbed specific agglutinins may be mechanically effected by means of the centrifuge, with results that coincide with those obtained by the standard method of test. Specific serological differences between meningo-

cocci, for example, may be determined by the centrifuge method. The technique is described.

By the elimination of the inconstant time factor in the flocculation phase opportunity is given for a closer analysis of specific absorption, and of the influence of various conditions upon both phases of agglutination.

The velocity of the absorption reaction is a function of the temperature at which it occurs, and the acceleration with increasing temperature is of the order of chemical phenomena. The absorption reaction proceeds most rapidly near the temperature of antibody destruction. The injurious effect of high temperature is revealed first in the serum; the antigen-antibody complex is not less sensitive.

The flocculation phase is also promoted by higher temperature, but lags far behind absorption, and consumes most of the time required for spontaneous agglutination.

The presence of an excess of antibodies greatly accelerates absorption and flocculation. The absorption reaction, under such circumstances, is ordinarily completed within a relatively short time.

THE EFFECT OF PULMONARY CONGESTION ON THE VENTILATION OF THE LUNGS.

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PLATES 1 TO 3.

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Clinical observations have shown that in patients with heart disease the vital capacity of the lungs is frequently less than normal, and that the decrease in the vital capacity bears a close relation to the development of dyspnea. As the tendency to dyspnea increases the vital capacity usually falls, and in patients who are bedridden on account of shortness of breath the vital capacity is rarely more than 30 per cent of the normal. This lowering of the vital capacity expresses the impossibility of increasing the depth of respiration in a normal manner, and under the stress of exercise such subjects are unable to raise the volume of pulmonary ventilation so as to bring about the necessary gas exchange between lungs and blood. The determination of the vital capacity has, therefore, considerable practical significance, since it serves as a guide to the general condition of the patient and often indicates changes in the functional condition of the circulation and respiration that are in harmony with the symptoms but are not necessarily suggested by changes in the physical signs.

The cause of this decrease in the vital capacity of the lungs in heart disease has never been adequately explained. In advanced cases it is, of course, due in part to pulmonary edema, pleural effusion, hepatic enlargement, and similar obvious factors; but in many cases the vital capacity is decreased without any physical signs which account for it, or with physical signs which are certainly insufficient to explain the degree of decrease.

The suggestion has been made (1, 2) that in such cases there may be an increase of pressure in the pulmonary circulation with engorgement of the alveolar capillaries of the lungs. As the alveoli are extremely vascular such a condition might produce a stiffening or *Lungenstarrheit*, in the sense of von Basch (3), which would interfere with their easy expansion and collapse in respiration. There might also be some protrusion of the distended capillaries into the alveoli and thus a decrease in the volume of residual air of the lungs. Since it is impossible to measure pulmonary arterial pressure in human beings, no statements can be made as to the degree of pressure increase which may develop in cardiac disease. From our observations upon cats in which the pulmonary veins have been obstructed, it is apparent that a high degree of pulmonary stasis may be produced without causing much actual increase in pulmonary blood pressure. The lungs act as a slightly elastic sponge, and are able to take up a vast amount of blood without significant pressure change. Furthermore, the normal right ventricle is unable to sustain pressures of any magnitude. We have attempted to increase pressure in the pulmonary artery through a clamp placed upon this vessel just central to its division. It is impossible to adjust such a clamp so as to provide a sustained increase in pressure of more than a few millimeters of mercury in the right ventricle of the cat without causing the death of the animal. We may, therefore, consider that when blood accumulates in the pulmonary circuit with a normal heart there is little attendant pressure increase, and that accompanying changes in ventilation are incident upon volume rather than pressure changes in this circulation.

In chronic cardiac disease with hypertrophy of the right ventricle it is conceivable that pressure in the pulmonary capillaries may reach abnormal levels, but it should be remembered that at the time we are interested in the question—the moment when the vital capacity begins to diminish—the heart muscle is in all probability beginning to fail, and, as a consequence, we are inclined to believe that even in human beings with considerable cardiac hypertrophy the rise in pressure in the pulmonary circuit accompanying a much increased blood volume in this circuit is not great. We are at present engaged upon experiments in which the pressure changes in the pulmonary

artery and capillaries resulting from pulmonary vein compression are being followed, and the statements just made must, therefore, be taken as expressions of our opinion at the moment, not as facts of experimental observation.

Clinical experience contributes several facts which are in harmony with the theory that the interference with the ventilation of the lungs, which shows itself by a decrease in the vital capacity, is due to a chronically increased filling of the pulmonary veins and capillaries. Thus, mitral stenosis is characterized by an early onset of the tendency to dyspnea and an associated low vital capacity. In aortic insufficiency, on the other hand, there is little tendency to dyspnea, and the vital capacity remains high until a relative mitral insufficiency develops and the pulmonary circulation is affected. Again, pleural effusions and pulmonary edema with râles are accepted signs of cardiac weakness. These probably find their cause in congestion of the pulmonary capillaries, and it is logical to suppose that their appearance is preceded by a phase in which the pressure may be very slightly higher than normal, but not yet sufficient to bring about exudation into the alveoli with eventual production of râles or the passage of fluid into the pleural cavity. Before either of these processes takes place there are no physical signs to indicate definitely changes in the pulmonary blood circuit. According to the theory suggested, however, even at this stage the vascular engorgement might interfere with the movements of the lungs and with the size of the alveoli, and cause a decrease in the vital capacity. If this theory is true, its practical significance will be easily understood, for the determination of the vital capacity will give the earliest and most accurate information which can be obtained about the pulmonary circulation. The earliest symptom in most cases of heart disease is an increased tendency to dyspnea and throughout the course of the disease this tendency is usually one of the best guides to the functional efficiency of the heart. It seems quite possible that the development of dyspnea depends largely on the condition of the circulation in the lungs, and, if such be the fact, any method which will help to throw light on one of the most obscure portions of the circulation will be of great clinical importance.

The problem which presents itself, therefore, as the result of the foregoing clinical observations, is to determine whether blood stasis

in the pulmonary circulation produces any effect on the ventilation of the lungs. If such a change does produce a limitation or reduction of the ventilation, then the theory that the reduction of the vital capacity of the lungs in heart disease is due to an increased filling of the pulmonary circulation receives considerable support. It is obvious that the question cannot be decided by the study of patients; the animal experiments to be described in the present communication were, therefore, designed in order to elucidate it.

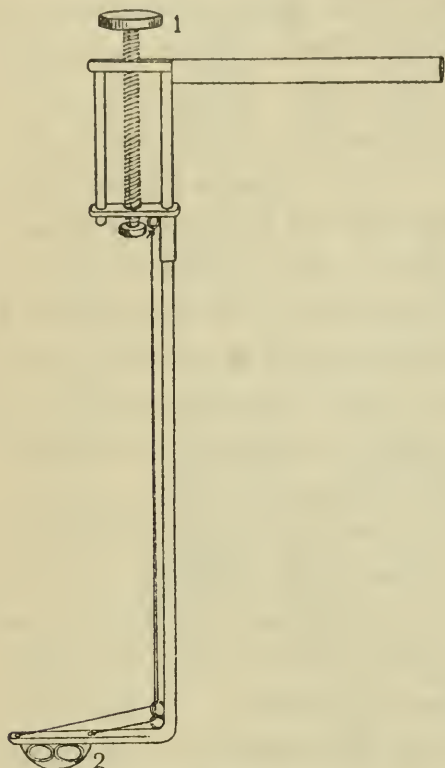
Technique.

The animals selected for study were cats. Because of the exquisite delicacy of the tissues to be studied and the relatively slight displacement in air space to be measured, certain experimental requirements were considered essential.

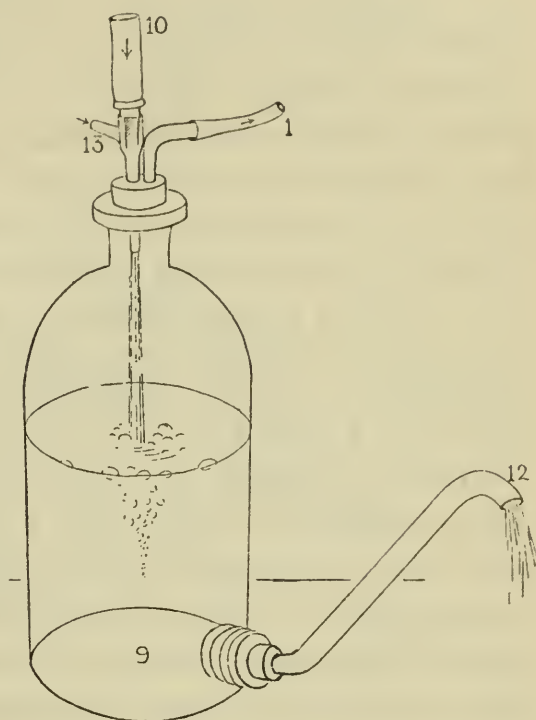
1. All spontaneous and reflex movements of the animal had to be eliminated. Even in the anesthetized animal compression of the pulmonary veins brings about active respiration, and such a reaction interferes with comparative measurements. Urethane anesthesia followed by curare obviates this difficulty.

2. A method had to be devised for compressing and releasing the pulmonary veins without any manipulation of the lungs. This was satisfactorily accomplished by the method described in detail previously (4). An oval section of the chest wall immediately over the heart is removed, the pericardium incised anteriorly, and the cut margins are reflected and sewed to the edges of the chest wall. With proper technique, the pleural cavity is rendered air-tight, permitting for hours independent respiration of the animal. With such a method the heart and great vessels lie exposed *in situ*, resting posteriorly on a pericardial sling. Advantage is then taken of the unusually high reflection of the pericardium in the cat, in which animal a ligature may be slipped about the pulmonary veins without in any way entering the mediastinum. The ligature is then threaded into the clamp as indicated in Text-fig. 1. The pulmonary veins, 2, represented in cross-section, can then be compressed and released by manipulation of thumb-screw 1. We have also found it possible to insert into the pulmonary artery a cannula of the type described by Schafer (5), in order to measure pressure in this artery during pulmonary vein compression.

All of these adjustments can be made after closing the thorax with the pericardium without the use of artificial respiration. In certain instances, of which Experiment 4 is an example, an intrathoracic cannula was inserted and connected either with a water manometer or with a delicately balanced volume recorder. Under such circumstances intrathoracic pressure or lung volume may be followed and the influence of blood in filling up thoracic space noted with accuracy.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Clamp for compression of pulmonary veins.

TEXT-FIG. 2. Apparatus for obtaining constant air pressure.

But in these instances the lungs are invariably collapsed away from the chest wall and are thus able to swell outwardly when they fill with blood. Such a condition is not permitted when the chest wall is properly closed and the lungs are well inflated against it.

3. The air delivered to the animal had to be at a relatively constant pressure and uniform rate of flow. This condition was satisfied by the apparatus illustrated in Text-fig. 2. The water flows down through tubing 10, under a constant head which is maintained by

a bottle suspended 9 feet above, kept full to overflowing. The water passes through an ordinary suction pump, draws in air through by-pass 13, and runs into an air-tight bottle, 9. Here air and water separate. Since the water level in the bottle is determined by the height of the top of the overflow tube, 12, the added amount of water passes out through this overflow tube, while the air is conducted out through tubing 1 to the animal.

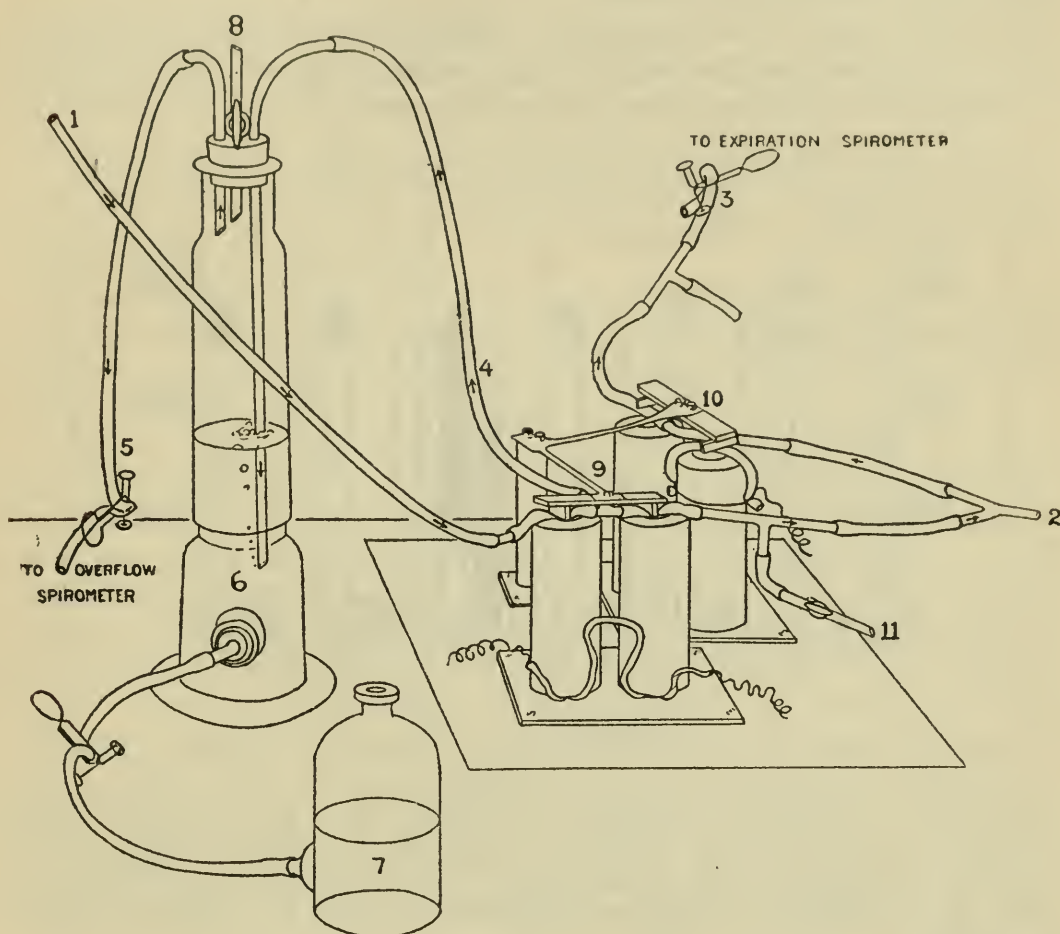
4. The apparatus had to be so designed as to register quantitatively any alteration in pulmonary ventilation. The artificial respiration machine indicated in Text-fig. 3 fulfills this need. It consists of two magnetic interrupters timed by a rotary switch to compress alternately the rubber tubing beneath them. The air passes to the animal from the bottle shown in Text-fig. 2 through an inspiratory tube, 1 (Text-fig. 3), to the cannula, 2, inserted into the trachea of the cat. During the period which constitutes inspiration, one hammer, 9, is raised, while a second hammer, 10, completely occludes the expiratory tube, 3. During expiration, the reverse situation exists, the expiratory air delivered through tube 3 being collected in a sensitive spirometer, known hereafter as the expiration spirometer.

Attention should be drawn to the shunt interposed between the two bars of hammer 9. This shunt, 4, by virtue of its position, operates only during inspiration and is designed to conduct all the air not accommodated by the cat's lungs through bottle 6 to another finely balanced spirometer, termed the overflow spirometer. If the available air space in the lungs is diminished, or the elasticity of the lungs becomes decreased so that they no longer expand as much under the same air pressure, more air will be collected in this overflow spirometer while less air will be collected in the expiration spirometer.

The construction and purpose of bottle 6 interposed in the overflow circuit deserve further comment. The air flows in through tubing 4, bubbles through the water in bottle 6, and escapes to the room through a stop-cock, 8, or, if this be closed and the clamp released, through tubing 5 to the overflow spirometer. Regardless of the avenue of escape of this air, however, the height of the water column in bottle 6, adjustable through container 7, determines the amount of air delivered to the animal. Thus, if the water column is high, little or no air will escape during inspiration into the overflow spirometer and

all the air will pass into the animal to be delivered to the expiration spirometer.

In brief, provision is made to collect all the air delivered to an anesthetized, curarized animal. This air reaches the animal rhythmically but under relatively constant conditions of pressure and flow.



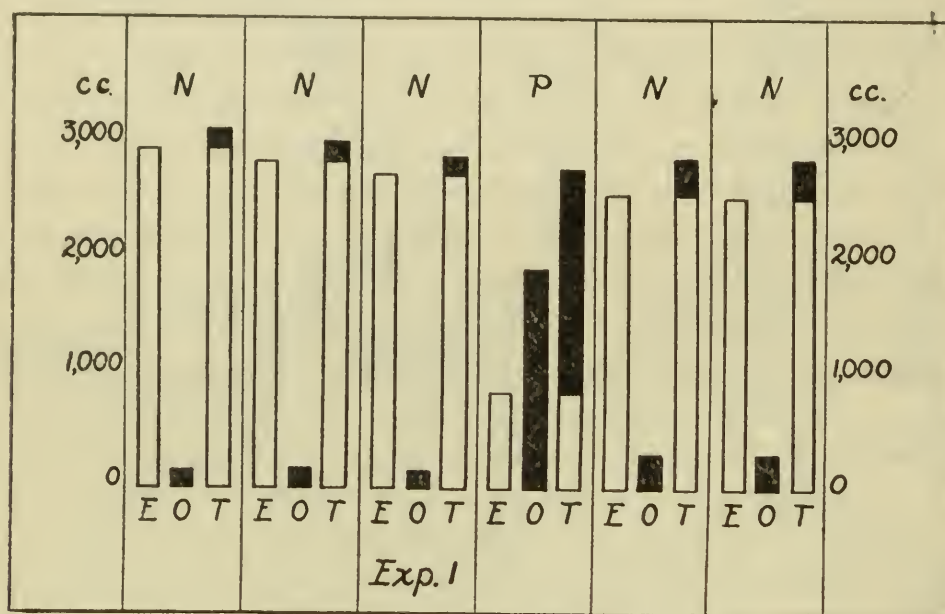
TEXT-FIG. 3. Magnetic interrupter for artificial respiration, with connections to spirometers.

It is collected in two spirometers and the total amount of air which these two receive will check closely in different periods of equal length. But if the pulmonary air space is decreased or if the movements of the lung tissue are restricted, the distribution of the collected air will at once change, a smaller amount being received by the expiration spirometer, and a larger amount by the overflow spirometer.

EXPERIMENTAL.

Experiment 1.—February 7, 1921. Cat; weight 2.8 kilos. 12.05 p.m. 28 cc. of 25 per cent urethane by stomach tube. 3.05 p.m. Operation on thorax completed. 3.30 p.m. 1 cc. of 2 per cent curare intravenously. Cardiometer and pulmonary vein clamp adjusted. Arrangements made for recording rate of respiration and femoral blood pressure.

The further course of this experiment is illustrated by Text-fig. 4 and Fig 1. Text-fig. 4 is a graphic representation of the six collection periods which comprised the experiment. There are, first, three normal periods, labeled *N*, in which air



TEXT-FIG. 4. Graphic representation of Experiment 1. Six 5 minute periods are shown. In this and the following text-figures *N* indicates normal period; *P*, period of pulmonary vein compression; *E*, air collected in expiration spirometer; *O*, air collected in overflow spirometer; *T*, total air collected by both spirometers.

delivered to the animal was collected in the expiration and overflow spirometers without any obstruction of the pulmonary circulation; then one period, labeled *P*, in which the pulmonary veins were compressed by means of the clamp shown in Text-fig. 1; and finally, two more periods, labeled *N*, prior to which the pulmonary vein clamp was released and the animal permitted to return to normal conditions. All periods in this and other experiments were 5 minutes in length, and in Text-figs. 4 to 7 the same method of representation is used. In every case the column marked *E* is the amount of air which actually entered the lungs of the cat and was collected in the expiration spirometer; the column marked *O* is the amount received in the overflow spirometer; and the column marked *T* is the

total amount of air delivered by the respiration apparatus during the 5 minute period, a total obtained by superposing the overflow column upon the expiration column.

Fig. 1 is a kymograph record of conditions during parts of the experiment. Section 2 covers the second period charted in Text-fig. 4. Tracing 1 is the cardiometer record made with the usual type of cardiometer transmitting through air to a large tambour. Tracing 2 is made by means of a second, rather stiff tambour and indicates the rate of respiration. A rubber tube (11, Text-fig. 3) on the inspiratory side of the respiration apparatus leads to this second tambour. Tracing 3 is the blood pressure record taken from the femoral artery by means of a mercury manometer. Tracing 4 is the base-line for blood pressure. Downward marks upon this line indicate 15 second intervals, but in this case are too irregular to be of value since the time-clock was out of order. Upward marks signal various events in the experiment.

During the first three normal periods, a constant amount of air was collected in the overflow spirometer but the total amount was slightly reduced. The change was, however, so much less than that occurring when pressure was applied to the pulmonary veins as to be negligible.

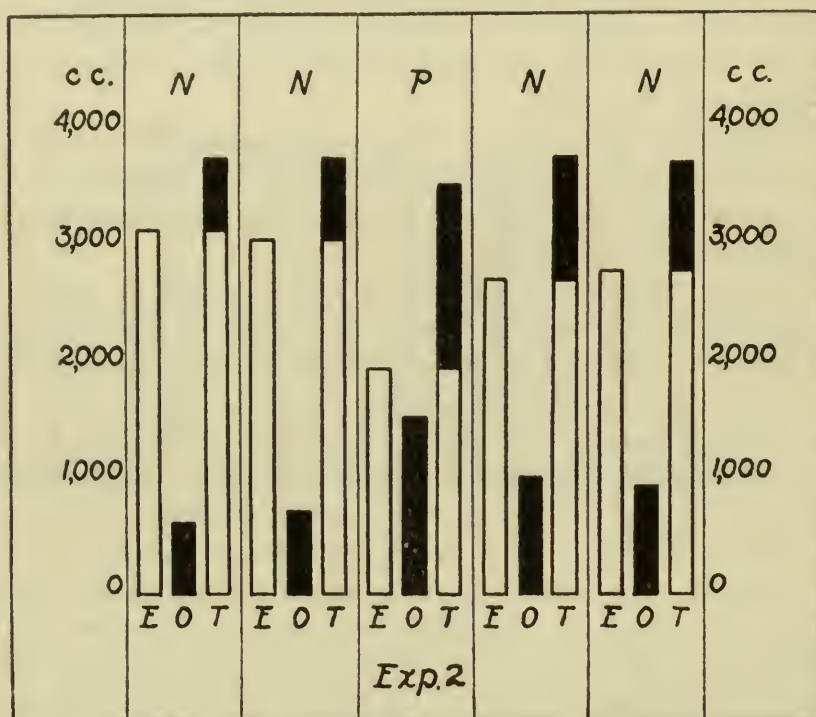
The first upward mark on the base-line in section 4, Fig. 1, indicates the beginning of pulmonary vein compression, brought about by tightening the clamp shown in Text-fig. 1. Between the second and third upward marks in this section of tracing, the air collection shown in *P*, Text-fig. 4, was made, and at the fourth upward mark the pulmonary vein pressure was released. The rise in the cardiometer record during this period shows a certain amount of cardiac dilatation; and the diminished movements of the writing point, a restriction of the individual beats. The fall in blood pressure indicates the degree of interference with filling of the left ventricle. The shift in distribution of the air during this period is clearly shown in *P*, Text-fig. 4. The great reduction in the amount of air collected in the expiration spirometer was due to pulmonary congestion incident upon the pulmonary venous obstruction, and the rise in the overflow collection merely accounts for the air which could not enter the lungs under the conditions imposed.

The two last normal periods *N* and *N*, Text-fig. 4, indicate an almost complete recovery after pulmonary vein release. The fact that this was not absolutely complete probably indicates a certain amount of intraalveolar extravasation which was not at once removed and which occupied space available for air in the first three normal periods. It is, however, clear that a very large measure of recovery did take place, and this means that the changed distribution of air noted in period *P*, Text-fig. 4, was due to intravascular blood.

The lungs, when removed at the close of this experiment, showed narrow longitudinal areas of congestion along the most posterior and consequently dependent portions of each lobe. The degree of change was comparable to that shown in Fig. 5, taken, it is true, from another experiment, but fairly indicative of the pathological changes occurring in experiments accepted by us as picturing the true relation of vascular filling and ventilation. On microscopic examina-

tion one finds epithelial desquamation with red cells and fluid in the alveoli making up the narrow area commented upon in the gross description of the lungs, and normal appearances in the rest of the tissue.

Experiment 2.—February 19, 1921. Cat; weight 3 kilos. 30 cc. of 25 per cent urethane. Operation and preparations for recording similar to those in previous experiment. Text-fig. 5 shows two normal periods, then a period of pulmonary vein compression, and finally, two more normal periods. The result is identical with that noted in Experiment 1. Fig. 2 is a graphic record similar to that in Fig. 1. Section 1 corresponds with the first normal period. Since pulmonary vein compression was produced rather slowly, section 3 has been



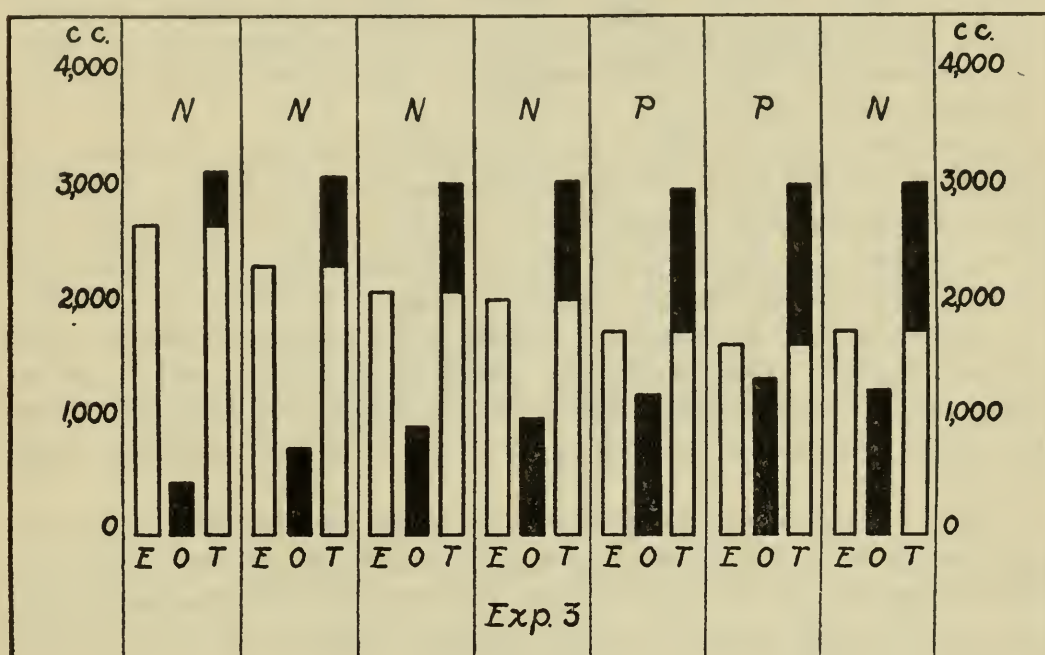
TEXT-FIG. 5. Graphic representation of Experiment 2. Five 5 minute periods are shown.

shortened to save space. The first upward mark on the base-line indicates the beginning of compression. Between the second and third, the air collection noted in *P*, Text-fig. 5, was made, and at the fourth mark the veins were released. Section 4 corresponds with the first normal period *N* after *P* in Text-fig. 5, and displays the condition of the circulation at that time.

On examination of the lungs an area of dark red congestion—not hard nor raised—was noted along the posterior surface of the right lung, and a few similar very small congested areas, chiefly along the margins of the lungs. It is interesting to note that the amount of overflow air in the last collection was somewhat less than in that just preceding, indicating a gradual tendency to return to normal.

In this case, the pulmonary vein clamp was tightened at 12.47 p.m., and collection period *P* took place between 12.56³⁰ and 1.01³⁰ p.m. The first normal collection period after clamping fell between 1.11¹⁰ and 1.16¹⁰ p.m. and the last one between 1.23 and 1.28 p.m.

Experiment 3.—February 19, 1921. Cat; weight 2.9 kilos. 29 cc. of 25 per cent urethane by stomach tube. A technique exactly similar to that of the two preceding experiments was used in this case except that two periods of pulmonary vein compression (*P, P*, Text-fig. 6) were employed. The result shows the effect of a progressively weakening heart and the production of extensive pulmonary edema. The heart was unusually large at the beginning of the experiment and



TEXT-FIG. 6. Graphic representation of Experiment 3. Seven 5 minute periods are shown, two of them in this instance being periods of vein compression.

there was more pericardial fluid than usual. Four normal periods were run and the volumes of air collected in the overflow spirometer showed an increase from 473 in the first period to 1,023 cc. in the fourth period. This increase was probably due to the development of pulmonary edema. In the third and fourth normal periods the volume in the overflow spirometer became more constant than it was in the first two, so that light compression of the pulmonary veins was tried in the 5th period.

The condition of the animal at this time is shown in section 5, Fig. 3. Poor adjustment of the cardiometer with leakage explains the atypical cardiometer record. Light compression caused a characteristic effect as noted in the first *P*, Text-fig. 6, and was followed in 9 minutes by a period of greater compression,

the second *P* in Text-fig. 6, and section 6 in Fig. 3. A greater degree of the typical change occurred, and that this was due in part to vascular engorgement, and not wholly to the progressive development of pulmonary edema, is indicated by the fact that release of the vein compression resulted in the last normal period (Text-fig. 6) in a slight return towards normal conditions. Section 7 in Fig. 3 indicates the condition of the animal at this time—a condition of progressively dilating heart and falling arterial pressure, the full extent of which was obscured by a clot which occurred near the end of the tracing.

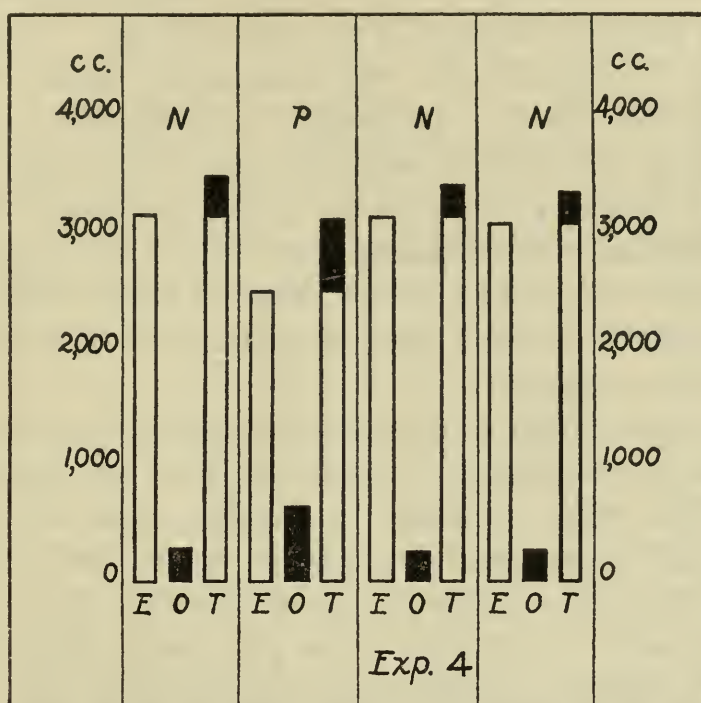
The lungs at the termination of this experiment were in bad condition, over two-thirds of the posterior surface being congested and dark red. There was, thus, extensive permanent damage. The experiment is published in order to display the course of events when pulmonary air space is steadily encroached upon by something more than intravascular blood. Such an animal is perhaps in a similar condition to that of the patient with reduced vital capacity and clear-cut physical signs in the lungs.

Experiment 4.—May 2, 1921. Cat; weight 2.7 kilos. 27 cc. of 25 per cent urethane by stomach tube. All preparations were exactly the same as in preceding experiments with one exception. The line of tracing numbered 2 in Fig. 4 indicates in this instance, intrathoracic volume changes. With the preparation completed in other respects, a glass tube was thrust through the chest wall and tied in place by a purse-string suture. Lung collapse at once occurred, probably bilateral. The intrathoracic cannula was connected with a delicate volume recorder constructed to write on a kymograph. On calibration it was found that a movement of the writing lever of 0.30 cm. recorded a volume change of 1 cm.

Text-fig. 7 is a chart of this experiment and shows a smaller but characteristic result during the period of compression, due probably to the fact that in this case the lungs were free to expand within the thorax, having been collapsed away from the chest wall, whereas in former instances practically all space occupied by blood was alveolar.

Section 1, Fig. 4, shows the condition of the animal during the first normal collection indicated in Text-fig. 7. Section 2 of the same figure is the period of pulmonary vein compression. At the first upward mark on the base-line, tightening of the pulmonary vein clamp was begun. Between the second and third upward marks the air collection period *P* of Text-fig. 7 took place, and at the fourth upward mark the pulmonary vein compression was released. The cardiometer and blood pressure effects are as in former experiments. The tracing of intrathoracic volume rises promptly, indicating a swelling of the lungs with blood, and the individual excursions of the recorder become less owing to less extensive movements of the lungs with each respiration. The maximum rise is equivalent to an encroachment on the intrathoracic space of 6.6 cc. and the individual excursions are diminished by an amount equivalent to 1 cc. Section 4, Fig. 4, represents the conditions during the final normal collection period of Text-fig. 7.

Fig. 5 is a drawing of the posterior aspect of the lungs from this animal. The broken lines lead to darkened sections which indicate the extent of a slight area of congestion—an area not great enough, however, to cause any effect upon ventilation, since it is noticeable that the overflow collections in the last two periods of Text-fig. 7 are not greater than that obtained in the first period of the experiment.



TEXT-FIG. 7. Graphic representation of Experiment 4. Four 5 minute periods are shown.

DISCUSSION.

As indicated at the outset, these experiments were designed to determine whether pulmonary congestion interferes with the entrance of air into the lungs. Congestion was produced by compression of the pulmonary veins at their entrance into the left auricle, and the effect on the air entering the lungs was determined by means of a sensitive artificial respiration apparatus which delivered a constant volume of air with each inspiration. Any obstruction to the entrance of air into the lungs of the animal was indicated by a smaller volume of air being collected on expiration and a larger volume being collected in a spirometer connected with the inspiratory tube, arranged to receive the "overflow" which did not enter the lungs.

The experiments fall into two groups—those in which there was essentially no permanent change in the lungs as a result of the manipulations, and those in which extensive pulmonary congestion with edema into the air passages was produced. In the former, illustrated by Experiments 1, 2, and 4, constriction of the pulmonary veins and the consequent overfilling of the pulmonary circulation caused definite interference with the entrance of air into the lungs. In the different experiments the quantitative effect naturally varied within wide limits. That the effect on pulmonary ventilation was the result of vascular change alone is indicated by the fact that release of pressure on the veins resulted in a practically complete return to the previous normal conditions. The normal appearance of the lungs at the end of the experiments is also evidence that the whole effect was a vascular one, which corrected itself as soon as the obstruction to the circulation was removed.

In the second group of experiments, typified by Experiment 3, compression of the pulmonary veins produced not only the simple vascular effect which appeared in the first experiments, but also caused extensive pulmonary congestion with edema into the air passages. Marked permanent damage was done to the lungs. In these experiments constriction of the pulmonary veins produced an interference with the entrance of air into the lungs, as it did in the first group of experiments, but release of pressure was not followed by a return to normal conditions. The changes in the lungs resulting from the passage of fluid out of the vessels into the pulmonary tissues and the air spaces were not relieved when the pressure in the vessels was allowed to return to normal. In these experiments the blood pressure in the greater circulation fell progressively throughout the observation, and it seems probable that the more extensive and permanent damage to the lungs was in part due to the development of cardiac weakness. This was especially evident in Experiment 3, in which the extensive exudation was undoubtedly due to the combination of circulatory obstruction and weak heart muscle. The results obtained in this second group of experiments are in no way opposed to those obtained in the first group. On the contrary, they supplement them. The two sets of experiments show that if the pulmonary veins are obstructed to such a degree that congestion of the pulmonary

vessels without exudation is produced, there results interference with the entrance of air into the lungs, which is relieved as soon as the obstruction is removed, but that if the circulatory conditions are such that exudation of fluid out of the vessels into tissues and air passages is produced, a permanent interference with the entrance of air into the lungs results.

There appear to be two ways in which pulmonary obstruction can act in order to encroach upon air space in the lung. First, the dilatation of the capillaries may actually take up alveolar space which air could occupy under normal alveolar conditions. This is a simple explanation of the observed change and readily accounts for the fact that less air entered the lungs on each inspiration. When the chest is closed and the lungs expanded so as to fill all the available space. it is clear that extra room which excess blood may occupy can only be obtained at the expense of the alveolar air space. The size of the lungs can only be changed, under these conditions, by pressures great enough to move the chest wall and diaphragm, and it is doubtful whether the right ventricle is capable of accomplishing such a result. When the chest is closed the lungs swell inwardly and large encroachment upon ventilation is the result. This change is illustrated by Experiments 1 and 2. When, however, the chest is open, blood may cause the lungs to swell outward, and under such circumstances the alveolar space may be very little changed and the amount of air entering the lungs not much altered. Experiment 4 illustrates just such a result and brings us to the second method by which pulmonary congestion may reduce the air space. It can be seen that in this experiment the expansibility of the lungs was diminished. The effect is, perhaps, as though the lung were erectile tissue rendered rigid and inelastic through vascular turgescence. Von Basch (3) suggested such an effect many years ago, and Experiment 4 is direct evidence of its existence but under abnormal conditions, the chest in this case being open. We are unable to say in what proportion these two factors operate to reduce ventilation in the closed chest, but are of the opinion that the first is the more important. It is possible that experiments now in progress, in which changes in capillary pressure are being measured, may throw light upon the situation.

We may now consider whether the facts derived from our experiments on animals afford any explanation of the conditions observed in patients with heart disease—conditions which were described at the beginning of this paper. In brief, it has been found clinically that patients with heart disease who become short of breath on exertion are unable to increase the depth of respiration, and that the vital capacity of the lungs is low. Experimentally it has been demonstrated that abnormal amounts of blood in the pulmonary circulation interfere with the entrance of air into the lungs. The clinical condition which has the closest analogy to our experiments is mitral stenosis. They are, indeed, essentially similar, for in mitral stenosis there is an obstruction to the flow of blood in leaving the left auricle, while in our experiments an obstruction was produced to entrance of blood into the left auricle. Both conditions, the clinical and the experimental, produce the same changes in the hemodynamics of the circulation in the lungs. In the early stages of mitral stenosis, when the obstruction to blood flow is slight, there is no abnormal tendency to dyspnea and no decrease in the vital capacity of the lungs. As the degree of stenosis increases, however, a tendency to dyspnea on exertion develops, and the vital capacity of the lungs is less than normal. Physical examination of the lungs reveals nothing to account for this interference with deep respiration. There is no exudation of fluid into the pleural cavities or into the air spaces, and thus no dullness on percussion, no alteration in breath sounds, and no râles. The conditions resemble those in Experiments 1, 2, and 4, in which there was engorgement of the pulmonary vessels with little or no exudation.

The inability to breathe deeply and the low vital capacity, which is one of the outstanding features of mitral stenosis at this stage, may well be explained by engorgement of the pulmonary vessels and lung rigidity, just as these factors explained the interference with the entrance of air into the lungs in our experiments. At a later stage of mitral stenosis, physical examination reveals râles in the air passages and fluid in the pleural cavities. These indicate actual exudation of fluid out of the vessels, and the conditions are similar to those typified by Experiment 3, in which extensive areas of pulmonary congestion were found. In the clinical disease, as well as in the experiments, the development of actual exudation depends on two factors,

the degree of obstruction to blood flow and the weakening of the heart muscle. At this period in clinical cases a still greater fall in vital capacity is found, and a greater tendency to dyspnea. The subject is unable to increase the depth of respiration enough to secure the degree of pulmonary ventilation necessary for aeration of the blood when the needs of the organism for oxygen are increased.

The relation of the conditions in the pulmonary circulation to the production of dyspnea is also suggested by other clinical observations. Mitral stenosis is the disease in which the most immediate effect on the pulmonary circulation would be expected, and dyspnea is an early symptom in mitral stenosis. Mitral insufficiency alone may not produce dyspnea for a long period of time, or apparently not until the heart muscle begins to weaken. The same is true of aortic insufficiency alone, but in association with a mitral lesion and involvement of the pulmonary circulation dyspnea begins to appear.

In aortic stenosis the situation is analogous to mitral stenosis, and when the pressure conditions are transmitted back to the left auricle and the pulmonary circulation, dyspnea becomes a prominent symptom. In all these diseases involving the heart valves the conditions are complicated by a coincident gradual degeneration or weakening of the heart muscle. No completely satisfactory analogy to this exists in our experiment for we were always dealing with a healthy myocardium. It was quite apparent, however, that the weakening of the heart in Experiment 3 acted in the same way as constriction of the pulmonary veins and served to increase the effects due to changes in the pulmonary circulation. It is not at all improbable that weakening of the myocardium alone may be enough to produce the same effects, and there is clinical evidence in harmony with this. Thus, in cases of myocardial weakness in later life the development of dyspnea on exertion is often the first symptom. This is usually accompanied by a fall in vital capacity, but at first there are no physical changes of importance in the lungs. Later, râles and pleural effusion appear. Dyspnea commonly develops early in such cases, while edema of the legs and hepatic enlargement come later. This suggests that in many cases of myocardial weakness the failure of the circulation takes place first in the pulmonary circuit. Clinical experience, as well as the experiments which have been described, indicates that in the

first stages there may be interference with the entrance of air into the lungs without the production of physical signs of lung involvement. At a later stage pulmonary edema and pleural effusion will take place.

SUMMARY.

1. A method is described for producing pulmonary congestion, together with what may be termed a differential spirometer method for studying lung ventilation.

2. The method utilized permits an approximately accurate prediction of degrees of pulmonary edema in the living animal, and suggests avenues of approach for the very difficult problems of pulmonary capillary pressure.

3. It is shown that intravascular blood can encroach markedly upon the pulmonary air space. Although the methods used in these animal experiments do not resemble vital capacity measurements in man, their result is so definite that their applicability to clinical conditions may be considered.

4. The similarity between the experiments described and certain conditions of cardiac decompensation, of which mitral stenosis is the best example, is pointed out.

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EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Kymographic record covering collection periods 2, 4, and 5 of Experiment 1. Upper line of tracing, marked 1, is the cardiometer record; tracing 2, the rate of artificial respiration; tracing 3, arterial pressure, mercury manometer, femoral artery; tracing 4, the base-line for blood pressure. Upward marks on this line signal events referred to in text; downward marks, 15 second intervals—inaccurate in this instance owing to defective clock. The tracing is reduced to about $\frac{1}{4}$ (it is actually $\frac{6}{273}$) of the original size.

FIG. 2. Kymographic record covering collection periods 1, 3, and 4 of Experiment 2. Lines of tracing similar to those in Fig. 1. The tracing is reduced to about $\frac{1}{4}$ (it is actually $\frac{6}{27\frac{1}{3}}$) of the original size.

PLATE 2.

FIG. 3. Kymographic record covering collection periods 4, 5, 6, and 7 of Experiment 3. Lines of tracing similar to those in Figs. 1 and 2. The tracing is reduced to $\frac{6}{27\frac{1}{3}}$ of the original size.

FIG. 4. Kymographic record covering collection periods 1, 2, and 4 of Experiment 4. Lines of tracing similar to those in Figs. 1 to 3, except that line 2 records intrathoracic volume changes and time marker records 5 second intervals. The tracing is reduced to about $\frac{1}{4}$ (actually it is $\frac{6}{27\frac{1}{3}}$) of the original size.

PLATE 3.

FIG. 5. The lungs of the animal used in Experiment 4. Dotted lines lead to area of congestion described in text.

76'

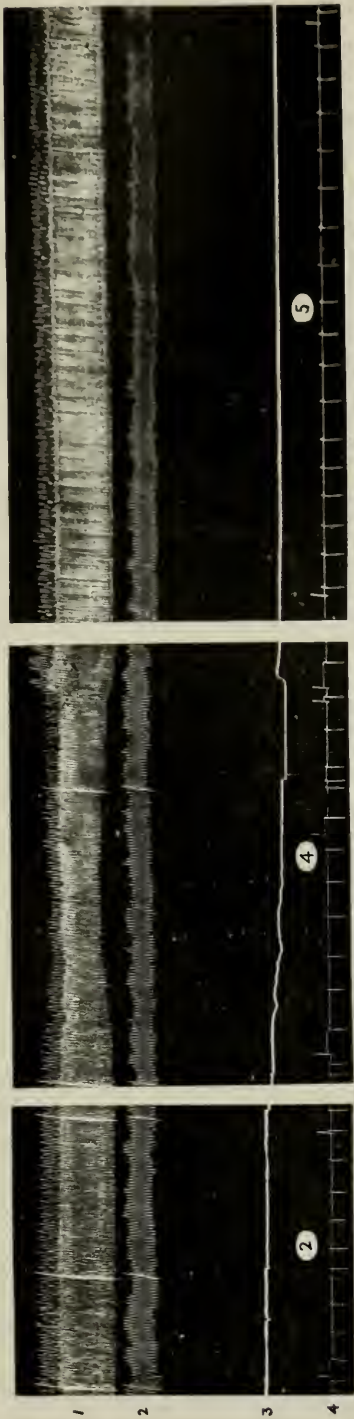


FIG. 1.

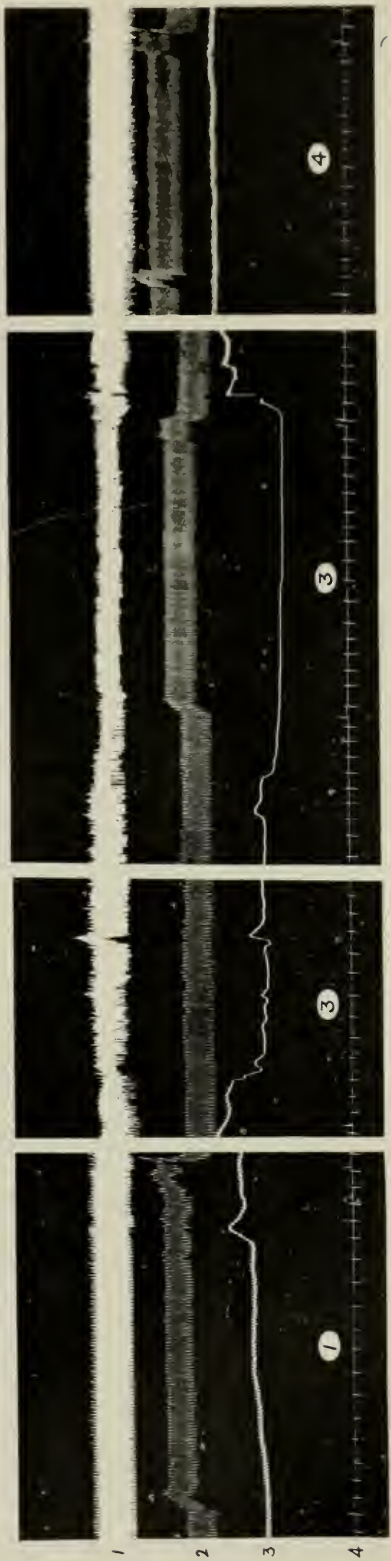
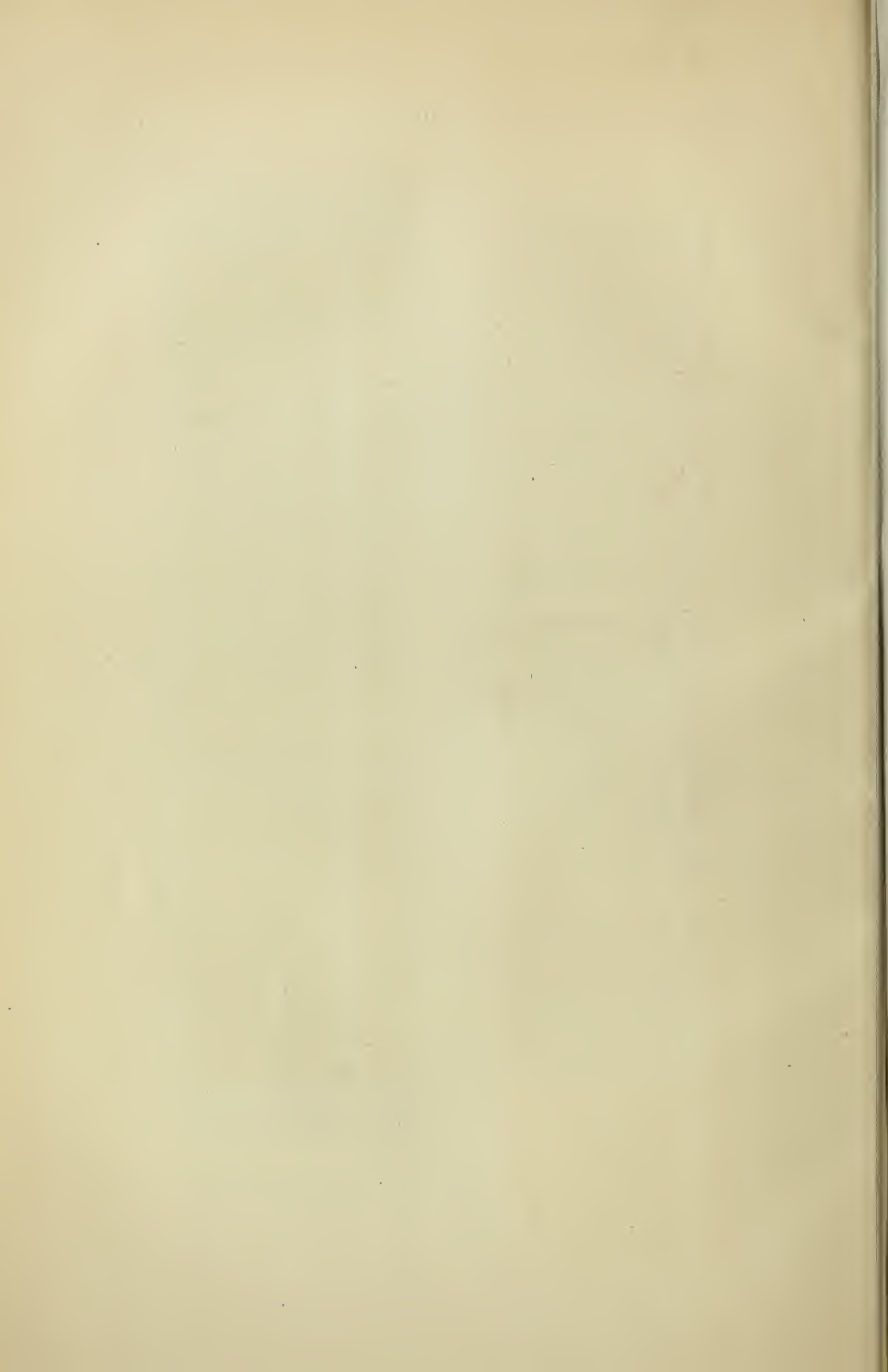


FIG. 2.

(Drinker, Peabody, and Blumgart: Pulmonary congestion.)



916²

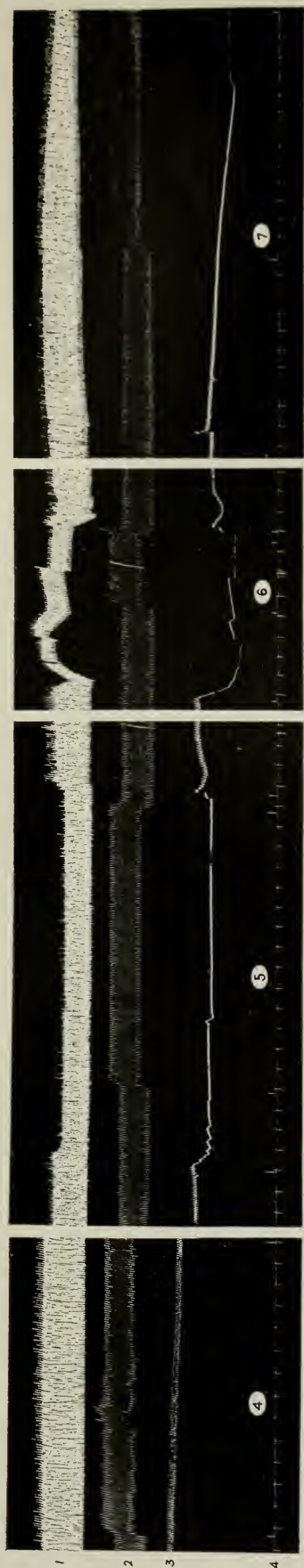


FIG. 3.

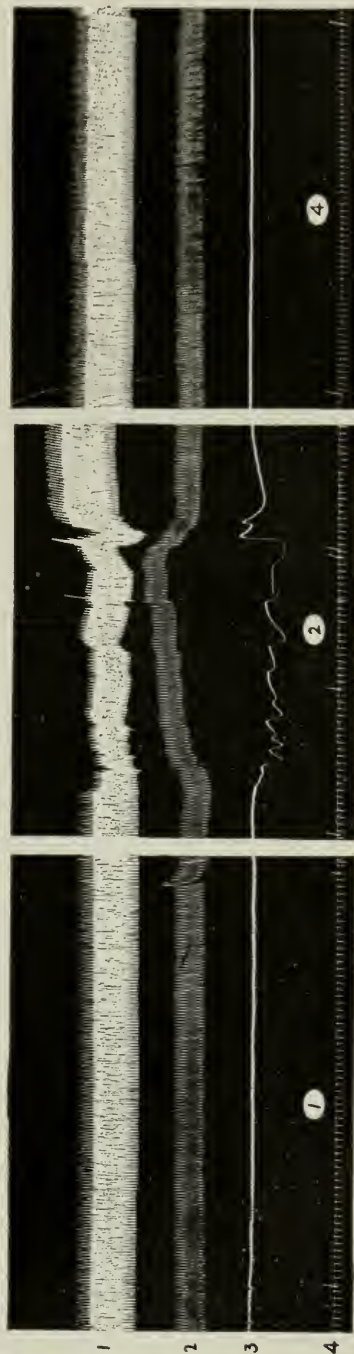
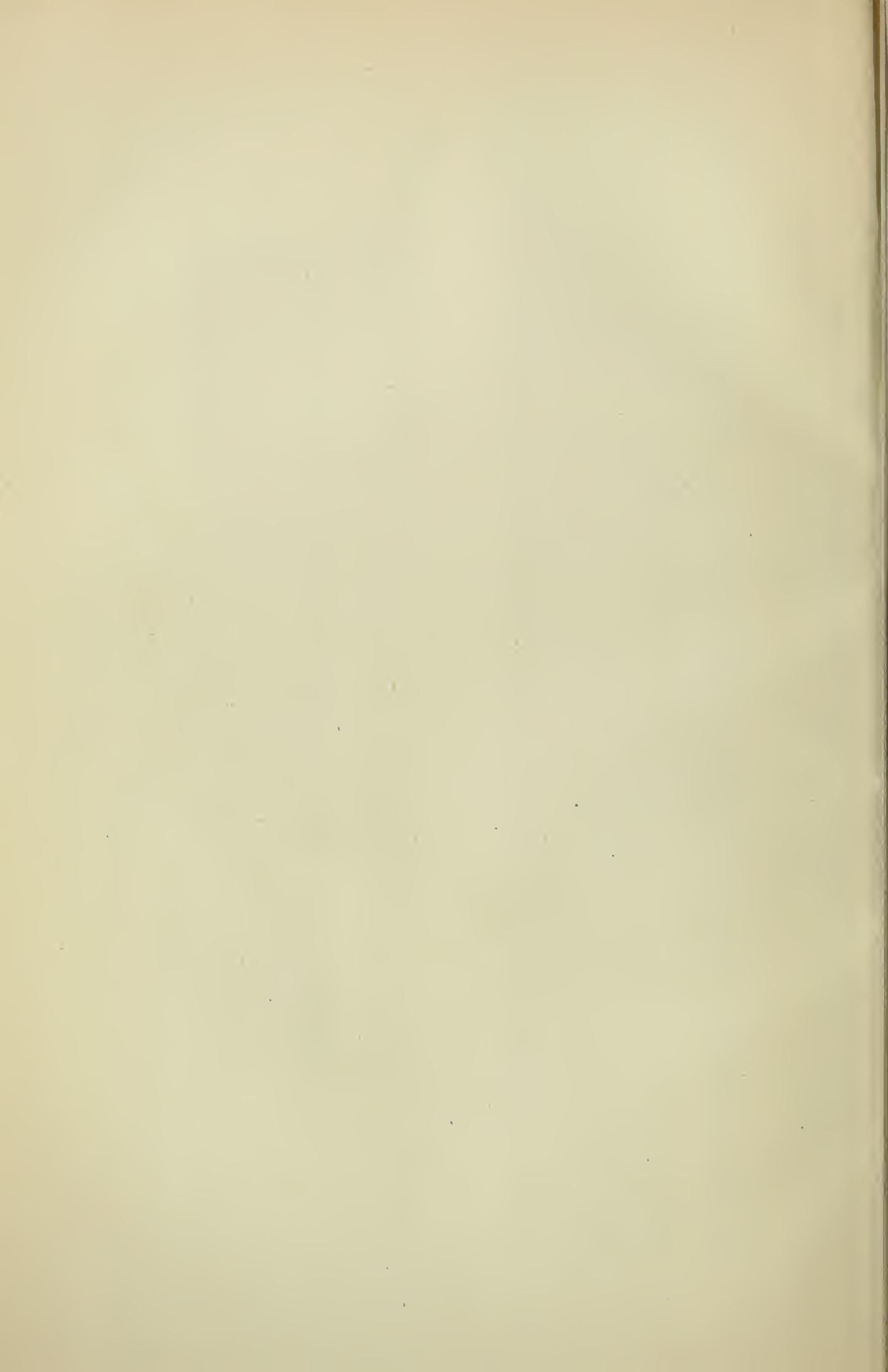


FIG. 4.

(Drinker, Peabody, and Blumgart: Pulmonary congestion.)



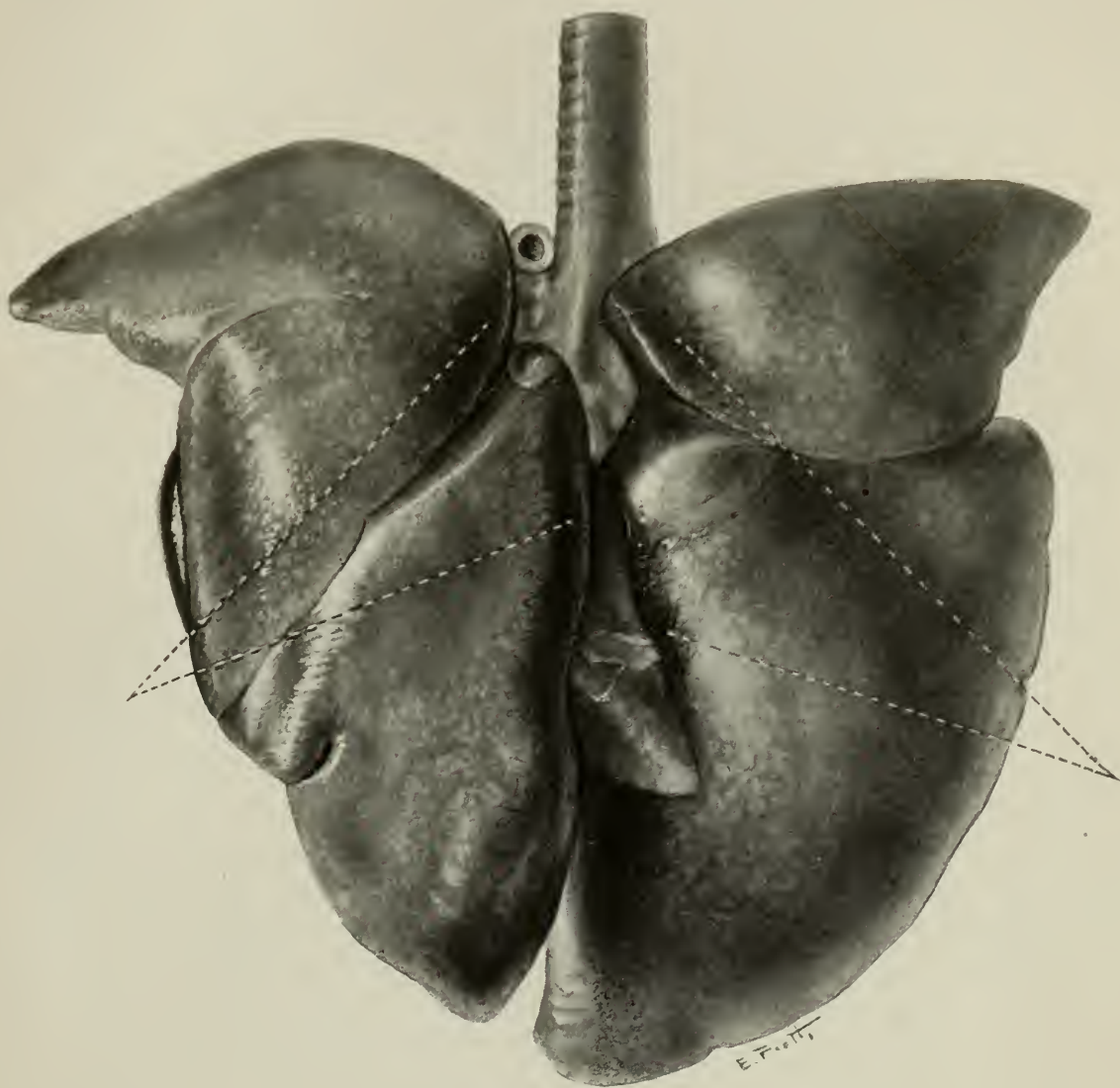
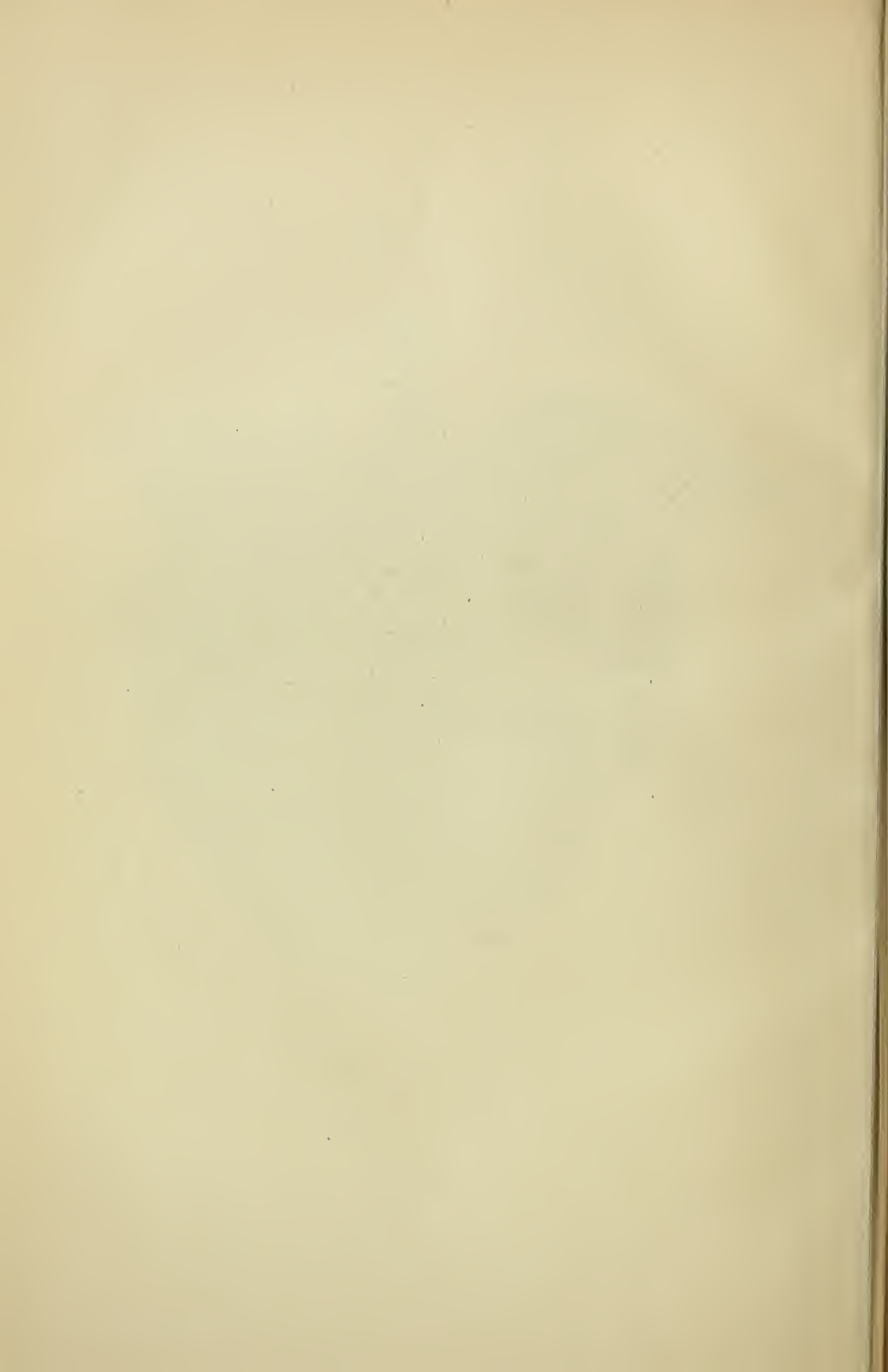


FIG. 5.

(Drinker, Peabody, and Blumgart: Pulmonary congestion.)



EXPERIMENTAL GENERALIZED ANALGESIA AFTER EXPOSURE TO SOME WAR GASES.*

By JOHN AUER, M.D.

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PLATES 4 TO 6.

(Received for publication, June 28, 1921.)

During a series of investigations, taken up at the instance of the Surgeon General of the Army, concerning the functional effects following exposure to dimethylsulfate and chloropicrin in a gaseous or finely subdivided state, it was noted accidentally that these two war gases reduced pain perception to an astonishingly low level. This analgesia was so profound in many cases that a laparotomy could be performed without any indication of pain and without markedly influencing the blood pressure. As this effect seems to be practically unknown, numerous experiments were carried out to test pain perception in gassed cats. The results justify the statement that the two war gases mentioned strikingly reduce pain perception in cats under the conditions which will be described.

The only reference in the literature of war gases bearing on this aspect which I have been able to find, is an article by Naiding.¹ This author had the opportunity of examining soldiers gassed with chlorine and phosgene, and states, among other observations, that numbers of them exhibited a general or localized loss of pain perception; thus,

* A preliminary note was published in the *Proceedings of the Society for Experimental Biology and Medicine* (Auer, J., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 104). The final publication was delayed by conditions beyond the control of the author.

This work forms part of some war gas investigations which began in 1917 and were undertaken at the suggestion of Dr. Simon Flexner and Dr. Henry D. Dakin. I am under special obligation to Dr. Dakin for numerous helpful suggestions concerning methods of gassing, precautions to be observed, and also for kindly supplying me with chloropicrin.

¹ Naiding, M. N., *Russk. Vrach*, 1917, xvi, 391.

he noted that the analgesia involved the skin of the whole body in some, while in others it was localized in the forehead, scalp, hands, or feet. There is no record that deep pain perception was tested.

Methods.

Cats only were utilized in this investigation for reasons of convenience. The gas chamber was of the ice box type with a cubic capacity of 200 liters. In two of the walls large glass windows were sealed so that the entire chamber could be readily inspected at any time; fogging of the glass panes was prevented by using a commercial soap sold for this purpose. One side of the chamber was formed by a completely removable door. Gas tightness was obtained by carefully filling all visible cracks in the wall of the inner chamber with putty and then applying several coats of white enamel. The door was made gas-tight by providing two soft rubber gaskets, one glued on the door and the other to the box. Before closure the contact surfaces were covered with vaseline. Complete contact was assured by four lever handles on the box which fitted in corresponding hooks on the door.

The cats were gassed in a wire cage, this container being placed on brackets in the chamber. The brackets were about 5 inches from the floor of the chamber so that the animal was at the same level as the window during the gassing period. Under the wire cage an enameled pan was placed to prevent soiling of the chamber.

The heat necessary for vaporizing the dimethylsulfate or chloropicrin was furnished by an electric hot-plate which could be set to reach 250°C. This plate was located in one corner on the floor and its switch was placed on the outside of the chamber. The hot-plate also served as a convenient means of maintaining the chamber temperature between 25° and 30°C. The temperature was read from a thermometer suspended near a window in the chamber.

As the gases employed were heavier than air, a small motor-driven fan was screwed in a corner of the chamber. This fan was kept in motion constantly throughout the experiment and prevented the gas vapors from settling to the floor.

The duration of gassing was kept constant for each of the various series of experiments. With dimethylsulfate the cats were gassed for 1 hour in the great majority of experiments. With chloropicrin the duration of gassing was either $\frac{1}{2}$ or 1 hour in most cases.

The concentration of gas chosen was such that death resulted in a majority of the animals within 1 to 4 days after gassing. This was determined by a series of trials and was as follows for the two gases: for dimethylsulfate 4.5 to 10,000 air; for chloropicrin 1 to 10,000 air.

The amount of liquid necessary to produce the required concentrations was readily calculated. Thus 20 cc. of gas were needed to give 1 to 10,000 concentration in a chamber of 200 liters capacity. As the molecular weight in grams of each liquid produces roughly 22 liters of gas, the weight of liquid required for 20 cc. of gas was determined and then translated into measure by volume. Curiously enough, the volumes of dimethylsulfate and chloropicrin necessary to produce

20 cc. of gas were identical: 0.087 cc. for dimethylsulfate and 0.088 cc. for chloropicrin. For practical purposes 0.09 cc. was considered the amount necessary for a 1 to 10,000 concentration. No corrections were made for temperature.

From these conditions it will be seen that the calculated concentration of the war gas would only be maintained for a short time, because condensation and droplet formation would begin shortly after vaporization. The gas concentration would then necessarily sink. This was not considered a drawback because the same conditions were maintained as far as possible throughout all the experiments; moreover, it is believed that this arrangement presents a closer approximation to conditions existing on the battle-field after the explosion of gas shells, than the ordinary procedure in which the desired gas concentration is drawn through the testing chamber.

Dimethylsulfate, $(\text{CH}_3)_2\text{SO}_4$, is a liquid at ordinary temperatures with a specific gravity of 1.32 and a boiling point of 188°C . Chloropicrin, or trichloronitromethane (CCl_3NO_2), is also a liquid and possesses a specific gravity of 1.69 and a boiling point of 112.8°C .

The chamber was evacuated by suction after the gassing period, the door of the chamber being perforated by two stop-cocks with $\frac{1}{4}$ inch lumen. These cocks were located in the upper and lower parts of the door respectively. The chamber gases were drawn off through the lower cock, while the upper one was open. Suction was furnished by the house vacuum system. Before the war gases were allowed to enter the vacuum system, they were passed through absorbents. For dimethylsulfate vapors 30 per cent NaOH wash bottles and soda lime tubes were used; for the chloropicrin gas, charcoal tubes were introduced into the system. The routine time allowed for evacuation was 30 minutes.

Thick rubber gloves were worn while removing the cage from the gas chamber and while cleaning it and the wire cage. The laboratory helpers were also encouraged to wear the regulation army gas mask (older form with nose clip) as a routine measure during the process of gassing; an hour's trial, however, invariably brought the conviction that this additional factor of safety was superfluous.

The precautions observed may seem excessive when it is considered that fractions of a cubic centimeter of the war liquids sufficed to give the desired gas concentration in the chamber, and that this amount completely vaporized in an average sized, well ventilated room would be entirely devoid of danger. The precautions, however, were made obligatory to counteract the depressing mental effect which war gas work exerted initially upon the helpers.

The methods employed in testing the analgesia produced by dimethylsulfate and by chloropicrin will be described in the section dealing with the experiments.

General Picture.

Before entering upon the subject of this paper it is necessary to give a brief description of the chief reactions which the cat exhibits during and after the period of gassing. While being gassed with dimethylsulfate in a concentration of 3 to

5 per 10,000 of air, the cats show a swiftly increasing irritation of the respiratory mucous membranes and of the conjunctiva. There is, first, winking of the eyelids, narrowing of the lid aperture, licking of the nose, and increased tear secretion. A little later the eyes are kept more or less closed. Sneezes and slight coughs now are noticed and a clear, watery saliva drips from the jaws. This watery saliva is soon replaced by a viscous secretion which hangs in gelatinous ropes from the angles of the mouth. At this stage the mouth is kept open most of the time, as apparently the nasal passages are obstructed. The respiration of the cat varies from 20 to 27 per minute and is not labored; towards the end of the gassing period most animals show short spells of polypnea, during which the tongue is protruded and the cat pants like a dog. Vomiting may occur during the last half of the gassing period or shortly after removal from the gas chamber. In general there is only a moderate restlessness at the start and then the animal lies or squats in a normal position on the floor of the cage. Certain animals, however, exhibit considerable excitement while being gassed, and this was more frequent among females than among males. The excitable cats were generally more severely affected by the same concentration than their more stolid mates. The mucous membranes of the mouth, and the tongue were usually red-pink throughout the gassing period, only occasionally was a slight cyanosis observable. It is worthy of note that most of the cats seemed astonishingly comfortable on the whole in the gas chamber.

After removal from the gas chamber the cats appeared to be in excellent general condition, but were disinclined to make any unnecessary movement; some muscular impairment was shown occasionally by a slight stagger on walking and by the short duration and weakness of resistance when examined. The animals preferred to crouch quietly in one place with head slightly extended and eyes closed. Inspection showed the jaws slightly open; slight or no salivation; moderately slow respiration, about 25 or 30 per minute, accompanied by rhonchi; expiration was slightly prolonged and active. On gently separating the lids the corneas generally exhibited a centrally located, oval, milky opacity, and the conjunctivæ and nictitating membranes were reddened and swollen. Auscultation revealed the lungs filled with snoring, groaning, clucking râles. These râles largely obscured the heart sounds. The blood pressure in the femoral artery was always at least 100 mm. Hg., generally more. From now on the course was usually progressively downward. The respiration became more and more labored; inspiration was slow and accompanied by a wide opening of the jaws; occasionally some retraction of the costal margins was observable during inspiration; expiration was usually prolonged and active. The mucous membranes were usually of a good color, but slight exertion of the animal sufficed to bring on a definite cyanosis. Death generally took place within 4 days, though some cats survived for months.

No food or water was taken by the cats which succumbed in 3 to 4 days; any attempt to swallow apparently led to choking.

Autopsy revealed a marked inflammation of the entire respiratory tract. The pharynx was reddened and occasionally covered with patches of yellow exudate;

the epiglottis was reddened, edematous, and occasionally covered with a gelatinous exudate. In some instances the tip of the epiglottis was occupied by a vesicle of edematous tissue. The entrance to the larynx may be a mere slit in a water-logged mass of tissue.

On opening the trachea and bronchi the entire mucosa was found covered by a grayish, gelatinous, thick membrane which extended apparently into the finer bronchioles. This membrane could be readily removed and formed a tough mass; frequently it was possible to obtain what seemed to be perfect tubular casts of a large part of the bronchial tree. The thickness of this pseudodiphtheritic membrane was often so great that the tracheal lumen on inspection seemed largely occluded, and some of the smaller bronchi were apparently completely plugged. Pulmonary edema was not frequent, and when present was remarkably localized, involving only one lobe or only a portion of a lobe.²

Chloropicrin gas in a concentration of 1 to 10,000 for 30 minutes or 1 hour causes symptoms which are in general similar to those obtained with dimethylsulfate. The main differences will appear in the following brief description.

As soon as the cat or cats are introduced into the gas chamber and before the chloropicrin has been heated, the animals wink rapidly and salivation begins. Within 1 minute ropy masses of saliva hang from the angles of the jaws; oftentimes clear, watery saliva drips over these glairy appendages. The salivation reaches a maximum and towards the end of the gassing period may have largely disappeared, as in dimethylsulfate intoxication. Retching and vomiting may occur early and is more frequent than with dimethylsulfate. During the early period of gassing a moderate restlessness is apparent. This is followed by a stage of quiescence during which the cat squats or crouches in the cage. Occasionally a short fit of restlessness intervenes. The respiration at the beginning of gassing is about 25 per minute; towards the end of the period it is generally shallower and faster, 50 per minute, with a slight active expiration. The prolonged active expiration seen with dimethylsulfate was absent, nor was opening of the jaws with inspiration such a prominent feature during and after chloropicrin gassing as when dimethylsulfate was employed. There was no evidence of marked asphyxia throughout the experiment, judging from the pink color of the tongue and mucous membranes of the mouth.

After removal from the gas chamber the cats were quiet and only slightly salivated. The respiration often was composed of short, weak inspirations followed by a sudden, sharp, active expiration. Auscultation showed the chest filled with coarse râles, which obscured the heart sounds. Muscular weakness was shown by feeble resistance to extension and by a disinclination to move. In the majority of instances death occurred within 48 hours after gassing with chloropicrin 1 : 10,000 for $\frac{1}{2}$ to 1 hour; a few cats, however, survived for months.

The autopsy exhibited a picture quite different from that produced by dimethylsulfate. The larynx and trachea were moist and pale, without any congestion

² Auer, J., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 106.

or membrane formation. Foam and fluid were generally found in the trachea; the lungs were large, heavy, and mottled with moist brownish areas and fresh hemorrhages. On closer examination these brown areas were seen to be areas of lung alveoli completely filled with fluid; the borders of the lobes were often emphysematous. On section, fluid poured from the cut surface as if from a saturated sponge. The parenchyma also often revealed numerous and large hemorrhages. On the surface of the section each bronchus and bronchiole was surrounded by a translucent, moist, gray zone of edema and the lymphatic trunks near the hilus were always engorged with clear fluid. This engorgement of the pulmonary lymphatics is observable at a time when the lung alveoli contain but traces of edema fluid, for example in cats killed after 15 minutes exposure to the gas. The edema was usually most pronounced in the lower lobes, though all lobes were involved. The degree of edema was often so great that moderate compression of the chest caused a gush of liquid from the trachea.

The diaphragm level was often abnormally low, due to a tonic contraction of its muscle fibers as indicated by the ridge formation of the muscle tissue at its tendinous insertion. This tonic contraction was also observed in cats in which a biopsy was carried out, and is perhaps ascribable to an *intra vitam*, partial rigor.

The heart muscle was usually brownish in color and flabby. On section, macroscopic hemorrhages in the left branches of the His bundle were frequently observed.

Analgesia.

Reduction of the capacity to perceive ordinarily painful stimuli was first observed during a tracheotomy performed to save an animal from the effects of a laryngeal stenosis. This animal had been gassed with dimethylsulfate 24 to 36 hours previously when suddenly it showed restlessness, the tongue and mucous membranes became bluish, the respiration was labored, and with each inspiration the costal margins now sank inwards. Tracheotomy was carried out at once and the symptoms were relieved.

Before describing the tests it is important to emphasize that all the manipulations were carried out with the greatest care and were always executed in a fixed way. The mild tests were invariably applied first and if they indicated analgesia, other procedures were cautiously attempted. As soon as a definite restlessness developed the test was discontinued. The cats were usually killed by injecting 10 to 15 cc. of ether into the trachea.

The tests were carried out while the cats were either lying or squatting on a table or when they were extended back down. A sharp, brief pressure of the pinna of the ear, the nasal septum, lips, the

skin of the body, the toe pads, and tail by means of a hemostat caused in typical cases no sign of pain. After the stimulus, the compressed ear was flattened against the skull, the head drawn to one side, the foot pulled away, or the tail twitched aside. In the normal cat a sudden moderate pressure upon the tail usually calls forth a short, strident yowl, an arching of the back, and a sidewise retreat while the animal spits a few times and glares with apprehensive resentment at the experimenter.

With the greatest precaution further tests were then made. It was found that the carotid or femoral artery could be exposed and freed from the accompanying nerves, that large flaps of skin could be resected, and that the tibialis anticus muscle and tendon could be dissected free, without causing any or only slight and indefinite evidences of pain. These evidences consisted chiefly of a slight restlessness and probably denoted discomfort rather than pain, for normal cats are not diffident about responding vocally to traumatic stimuli.

Still further experimentation showed that the peritoneal cavity could be opened in the midline; that, furthermore, a loop or loops of intestine could be delivered through the laparotomy wound and gently pulled, or that the parietal peritoneum could be rubbed with the finger without eliciting more than a slight general rolling motion of the body (Figs. 2 and 3).

However, marked stretching of a mixed nerve or of the mesentery, a strong electrical stimulation of an afferent nerve, all produced signs which could be interpreted as evidences of some perception of discomfort or even pain: the animal became moderately restless, the respiration quickened and deepened, and the blood pressure exhibited some elevation of its level.

The analgesia was generally well established within a few hours after gassing and within 24 hours reached a maximal degree. These animals were often in an apparently excellent condition, and Figs. 1 and 2 present an autographic record of how animals of this type fail to respond to ordinarily painful stimuli.

The record of Fig. 1 was furnished by a cat which had been gassed with dimethylsulfate in a concentration of 4.5 to 10,000 air for 1 hour. This animal was a male, originally weighing 4,150 gm., and had been

fed shortly before gassing. 26 hours after gassing he was in good general condition, though respiration was slow, slightly audible, and accompanied by some retraction of the costal margins; expiration was active and prolonged; the respiration rate was 32 per minute; his voice was hoarse. There was no obvious muscular weakness and the animal jumped with ease and coordination from the examining table to the floor. No food had been taken since exposure to the war gas, and his weight now was 3,760 gm., showing a loss of 390 gm. As soon as the animal was extended upon his back, the inspiration became more labored and snoring, and retraction of the costal margins increased in extent. The trachea was now exposed and partially slit transversely; the dyspnea was at once relieved, the inspiration now was unlabored and no longer accompanied by a snoring sound or by a costal retraction; expiration was also easier but was still moderately active. The trachea was surrounded by a zone of emphysema and the tracheal lumen was partly obstructed by a thick, gelatinous, membranous sheath, a part of which was removed.³ The operation developed not the faintest sign of pain; the animal was perfectly quiet and the tongue and mucous membranes had a better pink color than before. The left tibialis anticus muscle and left peroneal nerve were now completely dissected free and the nerve cut without any sign of pain; the cat rested quietly on its back and only the respiration showed a transitory acceleration in rate after section of the nerve. The animal was then prepared for recording the blood pressure in the right femoral artery. The section of the skin, the freeing of the artery from the crural nerve by blunt dissection, the placement of the nerve on a loop of thread, all caused no restlessness and the animal remained as quiet as if under the influence of an anesthetic. Stimulation of the intact crural nerve with the faradic current (300 mm. coil distance, two Daniell cells, Petzold coil, cable electrodes) induced a slight rolling motion of the animal accompanied by a blood pressure rise of 13 mm. Hg. (Fig. 1). Slight movements of the cat also caused elevations of the pressure by about 20 mm. Hg.

³ Removal of the tracheal membranous exudate is always dangerous because a section may be aspirated and plug the trachea and bronchi. For the same reason a transverse section of the trachea should never completely sever the tube of membranous exudate.

Exposure and severance of the left vagosympathetic nerve were painless. The cardiac vagus endings exhibited perhaps some reduction of sensitiveness, for a coil distance of 100 mm. was necessary to obtain the characteristic slowing of the heart (Fig. 1). The motor vagus endings in the esophagus reacted vigorously when the peripheral vagus was electrically stimulated with the coils 150 mm. apart. Stimulation of the central vagosympathetic trunk produced characteristic respiratory effects: slowing and deepening of the respiration associated with inspiratory stoppage broken by short vigorous expirations when a strong current was used (150 mm. coil distance; see Fig. 1). No respiratory stoppage in expiration was observed with the current strengths employed. The blood pressure exhibited the characteristic depressor type of fall when the left central vagosympathetic trunk was stimulated. The depressor portion of the vagosympathetic trunk was exceedingly sensitive, mere placement of the nerve stump on the platinum electrodes sufficed to cause a fall of 20 mm. Hg. (Fig. 1). The eye responded well to stimulation of the left central vagosympathetic trunk; with 250 mm. coil distance the nictitating membrane retracted slowly, but the pupil showed no dilatation; with 200 mm. the nictitating membrane retracted well and the pupil dilated strongly; on stoppage of the stimulation, the pupil contracted promptly to its original slit-like form. The tibialis anticus muscle exhibited no sign of fatigue when stimulated indirectly (peroneal nerve) with short tetanizing currents, 150 mm. coil distance, at 2 second intervals for 6 minutes. After 1 hour the rectal temperature was 35.7°C.

The depth of the analgesia was still further tested by opening the peritoneal cavity in the median line. There was no evidence of pain and the cat remained practically immobile. Insertion of the finger into the peritoneal cavity and gentle friction of the parietal peritoneum caused no resistance. The arteries of the intestine were bright red, while the veins were quite dark in color. The animal was then killed.

Quite similar results were obtained when chloropicrin was employed. The following abstract of a typical protocol, together with Fig. 2 giving the blood pressure record, will demonstrate this fact.

This cat was a non-pregnant female weighing 3,750 gm. and had

been fed previously; chloropicrin in a concentration of 1:10,000 was employed for 30 minutes. 23 hours afterwards the cat weighed 3,180 gm. but was in apparently good condition, though the respiration was moderately labored, with slightly snoring inspiration, about 44 to the minute, and was accompanied by an expiratory grunt. The expiratory grunt only became audible some time after expiration had begun. The tongue and mucous membranes were slightly cyanotic. The ordinary pressure tests yielded the same result which has been described in the dimethylsulfate animal. In this animal the dorsal position also increased the respiratory difficulty: the tip of the sternum now exhibited a definite depression with each inspiration and a moderate restlessness appeared. Tracheotomy was carried out at once and a fair amount of brownish fluid and foam escaped from the tracheal wound, which was increased by moderate compression of the chest and elevation of the foot of the board. As soon as the trachea had been slit the restlessness of the animal ceased, and it remained quiet; the tongue and mucous membranes became red without any trace of cyanosis. Some respiratory stenosis still existed, however, for the sternum still showed a slight depression with each inspiration. The right femoral artery and the left vagosympathetic trunk were also exposed. Fig. 2 shows the blood pressure record of this animal before, during, and after stimulation of the left peripheral and central vagosympathetic stumps; also before and after opening the peritoneal cavity and while gently rubbing the parietal peritoneum. The blood pressure curve of this animal reveals no significant changes while the parietal peritoneum was rubbed for approximately 10 seconds by the finger. The cat did not even move, for every movement is betrayed by a rise of pressure. The response of the cardiac vagus to electrical stimulation occurred with a weaker current in this cat than in the preceding animal; 200 mm. coil distance sufficed, while 100 mm. were necessary in the previous cat before a cardiac slowing was noticeable. The reflex respiratory effects in the chloropicrin cat after stimulation of the central vagus showed active expiratory stoppages and not inspiratory stoppages as in the dimethylsulfate animal. During these respiratory stoppages in active expiration foam and fluid were occasionally expelled from the trachea. The depressor nerve was not as sensitive in this animal as in the dimethyl-

sulfate cat. On the other hand, the chloropicrin cat exhibited a much greater sensitiveness of the dilator fibers of the cervical sympathetic, for a coil distance of 400 mm. sufficed to give a definite pupillary dilatation, while 250 mm. was the weakest current which was effective in the dimethylsulfate cat.

In this connection it must be mentioned that this cat exhibited definite hippus before tracheotomy had been carried out; the pupils dilated during inspiration and contracted well during expiration. The pupils responded to light and varied in diameter as the animal looked around (accommodation). The lid reflex was prompt. The arteries of the intestine were red, while the veins were moderately dark. The animal was killed later.

Autopsy of this cat showed a strong pulmonary edema; the lower lobes were full of fluid while the upper lobes contained less fluid but more foam and air.

These two abstracts and tracings (Figs. 1 and 2) reveal unmistakably that the capacity for pain perception, both superficial and deep, may be enormously reduced by exposure to the two war gases mentioned. The degree of analgesia exhibited by the two cats described above was not observed in all animals but in the great majority the presence of a well defined, undoubted analgesia could be readily established.

The duration of the analgesia could not be determined with accuracy because most of the cats succumbed within 4 days. After chloropicrin intoxication a number of cats exhibited, about 5 days after gassing, outspoken signs of pain perception when mild pressure tests were applied to the tail. The reaction was so definite that no further tests were made. The same reestablishment of pain perception was also noted in a chloropicrin cat which survived for 5 months. After dimethylsulfate, on the other hand, the analgesia may still be present to a remarkable degree even after the lapse of 6 months. Fig. 3 illustrates the one instance I have observed.

This cat was a male weighing 4,250 gm. originally, and had been subjected to the effects of 4 parts of dimethylsulfate to 10,000 of air for 1 hour. The effects were severe and the animal did not show a change for the better until 7 days after gassing. 10 days after gassing, food was taken more regularly and the animal moved about in a practically normal fashion. 6 months after gassing, the animal

was still alive though markedly emaciated, and weighed 2,890 gm., which represents a loss of 1,060 gm. The emaciation was not due to failure to eat, for the animal ate heartily. There was no definite muscular weakness discernible and he walked and jumped on occasion like a normal animal. The nose showed a yellowish fluid discharge. Both corneas were spotted with a central staphylomatous leucoma and synechias stretched between the upper pole of the iris and the cornea of each eye. The respiration was slow and easy, with slightly prolonged expiration, and the rate was 40 per minute. Moist, throaty sounds often accompanied the respiration. The usual tests were carried out and no definite signs of pain perception were elicited. Fig. 3 represents the blood pressure curve of the animal while being tested for deep pain perception. Both vagosympathetic trunks were intact and no tracheotomy was performed in this cat. It will be observed that a pull on the crural nerve caused only a slight short rise of pressure which was considerably less than that obtained when the animal was slightly restless just preceding this test. Opening of the abdominal cavity caused no change in the pressure level worth mentioning nor did restlessness occur. That the procedure had caused reflexly effective afferent impulses is seen, however, in the rise of the pulse rate from 140 to 160 and in the increase of the respiratory rate (25 to 32 per minute). Introduction of the finger into the abdomen caused a short rise of the blood pressure from 126 to 148 mm. Hg. and was associated with slight general movements. These reactions surely indicate that sensory impulses reached the higher centers, but from the general behavior of the cat it seems equally certain that these sensory impulses were perceived as uncomfortable sensations rather than as pain sensations. The cat was then killed by means of ether.

DISCUSSION.

The observations reported on preceding pages indicate clearly that cats gassed with chloropicrin or dimethylsulfate under the conditions mentioned do not react normally when subjected to stimuli which are painful under ordinary conditions; operative interferences may be undertaken, involving the skin, the deeper interfascial structures of the leg and neck, or the peritoneum even may be incised and its

parietal leaf rubbed, without eliciting the ordinary signs of pain. In other words, gassing cats with one of these two war liquids endows the treated animals with a marked degree of analgesia both for superficial as well as for deep pain stimuli.

The question now arises how this experimental analgesia is produced, whether there is any single factor at work which may explain this curious effect of at least two, chemically different war gases. There are four possibilities to be considered: (1) anesthesia of the sensory nerve endings of the skin; (2) blocking of sensory impulses in the nerve trunks; (3) that the analgesia does not exist in reality but is simulated by the fact that motor weakness prevents expression of the normal reaction to pain stimuli; and (4) that the higher centers are largely unable to perceive pain stimuli. It must be stated at once that some of these factors can be excluded with considerable certainty, yet perhaps all may be involved to some degree in the causation of the final result; on the whole, however, one factor appears to play a dominant rôle. The various possibilities will now be discussed briefly.

It might be assumed that the receptive sensory endings in the skin are damaged by the war gas vapors which condense to some extent at least in the fur of the animals. Such a condensation apparently does take place, for the behavior of fleas, with which most of the cats were infested, indicates this. Shortly after gassing the fleas always became restless, left the deeper portions of the fur, and climbed to the extreme tip of the longer hairs where they maintained a precarious position with difficulty until increasing weakness caused them to fall or jump feebly to the floor of the gas chamber where they died. The experimental evidence at my disposal does not permit any statement as to whether or not the two war gases produced the postulated skin anesthesia, but the facts already submitted show definitely that such an anesthesia fails to explain the presence of analgesia to deep pain stimuli (stimulation of nerve trunks, opening of the peritoneal cavity; see Figs 1 to 3). The localized analgesia observed in gassed soldiers by Naiding would seem to indicate, however, that under certain conditions some war gases may destroy the functional activity of the sensory skin endings. It is possible that this local action was reinforced by a systemic effect, for Naiding states that

80 per cent of his patients complained of pain in the chest, asthma, or coughing.

The second possibility was a blocking of sensory impulses in the nerve trunks subsequent to the inhalation of the war gases. The experiments and tracings give no support to this supposition. In numerous instances typical analgesia was present when examination revealed a prompt knee jerk, a pupil responding normally to light, accommodation and sympathetic nerve stimulation, and prompt, effective, medullary reflexes when the central vagus and depressor nerves were stimulated (see Figs. 1 and 2). These results would be impossible if conductivity in the nerve trunks were strongly reduced. Whether or not, however, a qualitative blocking for pain stimuli existed cannot be discussed profitably in the present state of our knowledge.

The third possibility considered the analgesia as non-existent in reality and assumed that peripheral motor weakness prevented the normal response to pain stimuli. This explanation is absolutely disproved by the fact that stimulation of a motor nerve produces normal muscular contractions, and by the further fact that the gassed muscle, for example the tibialis anticus, may show little if any greater fatigability than a normal muscle when stimulated directly, or indirectly through the severed peroneal nerve, the muscle being left *in situ* with blood and motor nerve supply intact.

There remains for consideration the fourth possibility, that the higher centers are largely unable to perceive pain stimuli as such, and this assumption satisfactorily explains all the observed facts. The foundation for this belief has already been presented by exclusion in the discussion of the three explanations of war gas analgesia, all of which placed the cause in the periphery. The dismissal of these peripheral causes as inadequate or inadmissible compels the view that the cause must be located in the higher centers. The higher cerebral centers, therefore, after gassing with dimethylsulfate or chloropicrin are apparently so altered that ordinary pain stimuli are no longer recognized as such, and this alteration does not appreciably interfere with execution of most of the basic reflexes which link the functional organism into a unit.

What the nature of this alteration may be can be inferred to some

extent after determination of the active agent which causes these specific changes in the higher nerve cells. In the search for this agent it might be assumed that the war gases producing analgesia perhaps show similar chemical groupings or structures and that this is causally related to the production of analgesia. There is no definite basis for this supposition beyond the fact that the three gases which have been described as producing analgesia, dimethylsulfate ($(\text{CH}_3)_2\text{SO}_4$), chloropicrin (CCl_3NO_2), and phosgene (COCl_2), may all be considered methane derivatives. The observation, however, that generalized analgesia may persist for 6 months after gassing (see Fig. 3) renders the view quite improbable that the war gas itself is the agent directly producing the analgesia. As far as I know there is no volatile chemical substance, excluding sublimation of the heavy metals, which can exert an effect in the body lasting for months after a single administration. It is therefore necessary to seek among the secondary effects of war gases for the common agent which produces analgesia. This common agent I believe to be a general, persistent, low grade asphyxia. The reasons are as follows:

It was noted that moderate exertion of short duration changed the pinkish buccal mucous membranes of the resting cat to a bluish cyanotic color; a mere change of position, for example extension on the back, often sufficed to bring on a notable cyanosis associated with restlessness, dilatation of the pupil and increased dyspnea; all of these undesirable results generally could be relieved by tracheotomy. Autopsy of these animals revealed the pulmonary air passages largely filled with a membranous exudate or with edema fluid. It therefore seemed clear that during life the lung ventilation barely sufficed for the vital needs of the organism, and that a small additional respiratory difficulty or increased production of CO_2 (struggles) brought on an obvious asphyxia. Furthermore, there seemed to be a definite association between the presence of marked analgesia and the functional capacity of the lung. Thus a number of chloropicrin survivors exhibited normal reactions to moderate pressure stimuli about 6 days after gassing. These responses were so clear that further tests were not deemed justifiable and were therefore omitted. After killing these cats with chloroform, examination showed the lungs to be in good functional condition; the color was pink, they

collapsed normally on opening the chest cavity, section revealed no trace of edema, and there was no stenosis of the air passages. The only marked abnormality was atelectasis of one or more of the upper lobes. In these cats, therefore, pulmonary ventilation of the blood was practically normal and no asphyxia existed. On the other hand, a dimethylsulfate survivor exhibited a typical analgesia 6 months after the date of gassing (see Fig. 3). Autopsy of this cat exposed a voluminous lung which collapsed but slightly on opening the chest; its borders were emphysematous and section disclosed the pulmonary passages including the trachea, apparently completely filled with a yellowish, semifluid, gelatinous exudate resting on a tough membranous base. The mechanical obstruction in the trachea, bronchi, and bronchioles was such that pulmonic ventilation seemed impossible, and yet no signs of respiratory distress were observed while the cat was walking about; indeed the cat seemed quite comfortable for it often raised its tail to a vertical position, rubbed itself against the observer, and made moist, purring sounds. In this cat a low grade, chronic asphyxia surely existed.

The various facts presented above legitimize the inference that a systemic, low grade asphyxia of pulmonic origin is a primary factor in creating and maintaining the generalized analgesia described in this paper. Other factors probably also contribute to the final result. Among these, inspissation of the blood⁴ due to edema, salivation, vomiting, and deficient fluid intake, and cellular changes⁵ in various parenchymatous organs of primary or secondary origin, may be of some value. The formation of methemoglobin apparently does not take place to any extent with the doses of dimethylsulfate and chloropicrin employed in these experiments. The most asphyctic blood readily turned to a bright red color when lightly shaken with some Ringer solution; moreover, Gildemeister and Heubner⁶ report

⁴ A careful study of various blood changes after chloropicrin, phosgene, and chlorine inhalations in the dog has been made by Underhill (Underhill, F. P., *The lethal war gases: physiology and experimental treatment*, New Haven, 1920).

⁵ See Gildemeister, M., and Heubner, W., *Z. ges. exp. Med.*, 1921, xiii, 325, for a description of cellular changes after chloropicrin.

⁶ Gildemeister, M., and Heubner, W., *Z. ges. exp. Med.*, 1921, xiii, 315.

only doubtful spectroscopic signs of methemoglobin in the blood of cats, even when the doses of chloropicrin were so large that death resulted within 15 to 18 minutes.

The analgesia described in the preceding pages may be of some practical importance in the human subject. In gassed soldiers, for example, where surgical intervention is necessary, it is quite probable that little or perhaps even no general anesthetic may be necessary. This would be desirable because it removes, for some patients at least, the danger of a general anesthesia by means of ether or chloroform in a partially asphyctic individual. Another benefit would be a conservation of the immediately available supply of anesthetics, a factor of some importance for stations in the field of operations where transport facilities are never ideal.

SUMMARY.

Cats gassed with dimethylsulfate or chloropicrin in such concentration that death generally results within 4 days, usually exhibit a marked generalized analgesia, both superficial and deep.

Gassed cats react with no obvious sign of pain to operative interferences, including laparotomy and gentle friction of the parietal peritoneum.

The analgesia develops within a few hours after gassing, and reaches its maximum in about 24 hours. With dimethylsulfate the analgesia may persist for 6 months; with chloropicrin practically normal sensitiveness has been observed 7 days after gassing.

This analgesia is considered to be caused and maintained largely by a general, low grade, tissue asphyxia which is chiefly of pulmonic origin.

EXPLANATION OF PLATES.

The blood pressure curves were recorded from the right femoral artery with a mercury manometer. The anticoagulant for the tubing was one-half saturated sodium sulfate solution.

The time marker registers 4 second intervals, and the time line represents also the line of zero pressure.

The broad white bands on the line below the time record indicate the time and duration of nerve stimulation.

l.p.v. indicates left peripheral vagosympathetic trunk; *l.c.v.*, left central vagosympathetic trunk.

Electrical stimulation: Petzold induction coil with two Daniell cells, platinum point cable electrodes; numbers refer to distance in millimeters between primary and secondary coils.

PLATE 4.

FIG. 1. Gray and white cat, male; weight 3,760 gm. Gassed with dimethyl-sulfate 4.5 to 10,000 for 1 hour. Tracing obtained 26 hours after gassing. Tracheotomy performed. Left vagosympathetic nerve cut during experiment; right intact. In this cat the abdominal cavity was later opened and the parietal peritoneum gently rubbed without causing any pain.

PLATE 5.

FIG. 2. Maltese cat, female; weight 3,180 gm. Gassed with chloropicrin 1 to 10,000 for 30 minutes. Tracing made 23 hours after gassing. Tracheotomy necessary because of pulmonary edema. Left vagosympathetic nerve severed before record was taken. Slight rolling motions at the beginning of the record are betrayed by the two moderate pressure elevations near the beginning of the curve. Note very slight effect of rubbing the parietal peritoneum for 12 seconds towards the end of the tracing.

PLATE 6.

FIG. 3. White and gray cat, male; weight 4,250 gm. Gassed with dimethyl-sulfate 4 to 10,000 for 1 hour. Tracing obtained 6 months later; weight at this time 2,890 gm. Both vagi were intact; no tracheotomy was necessary. Opening of the peritoneal cavity and insertion of a finger caused only a slight general movement associated with a rise of 20 mm. in blood pressure. The blood pressure, pulse rate, and respiratory rate were the same at the end of the tracing as at the beginning: 130 mm., 165 heart beats, and 30 to 32 respirations per minute.

Feldavit

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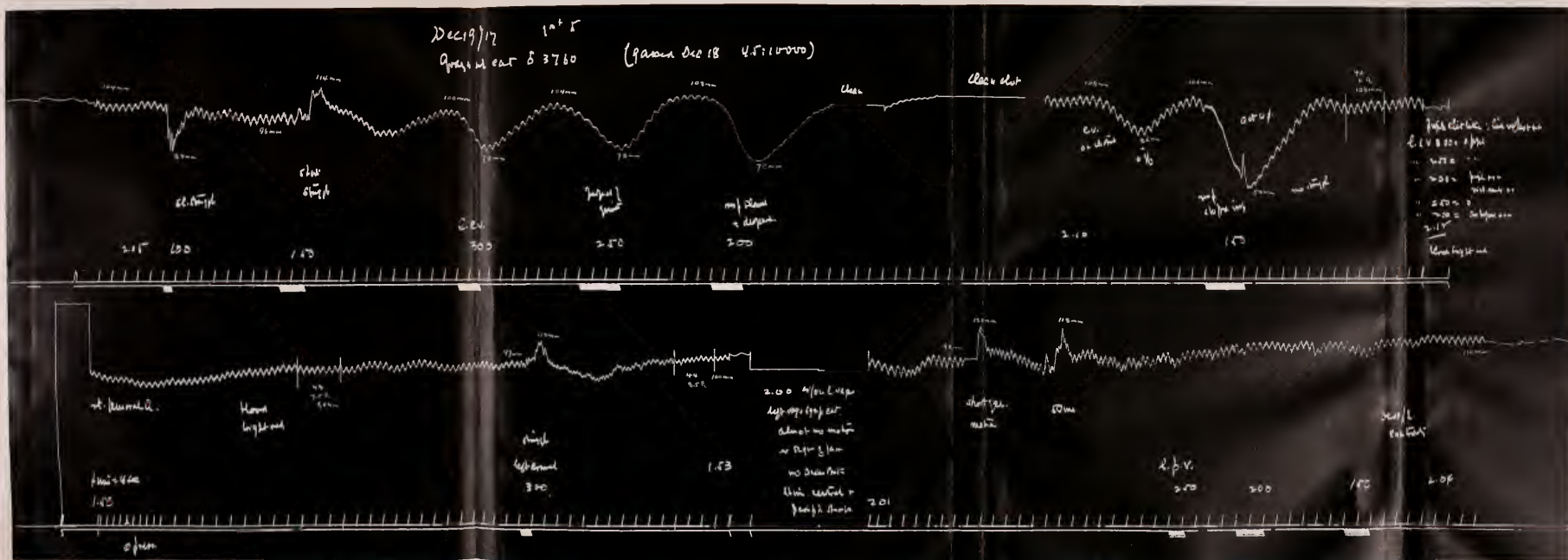
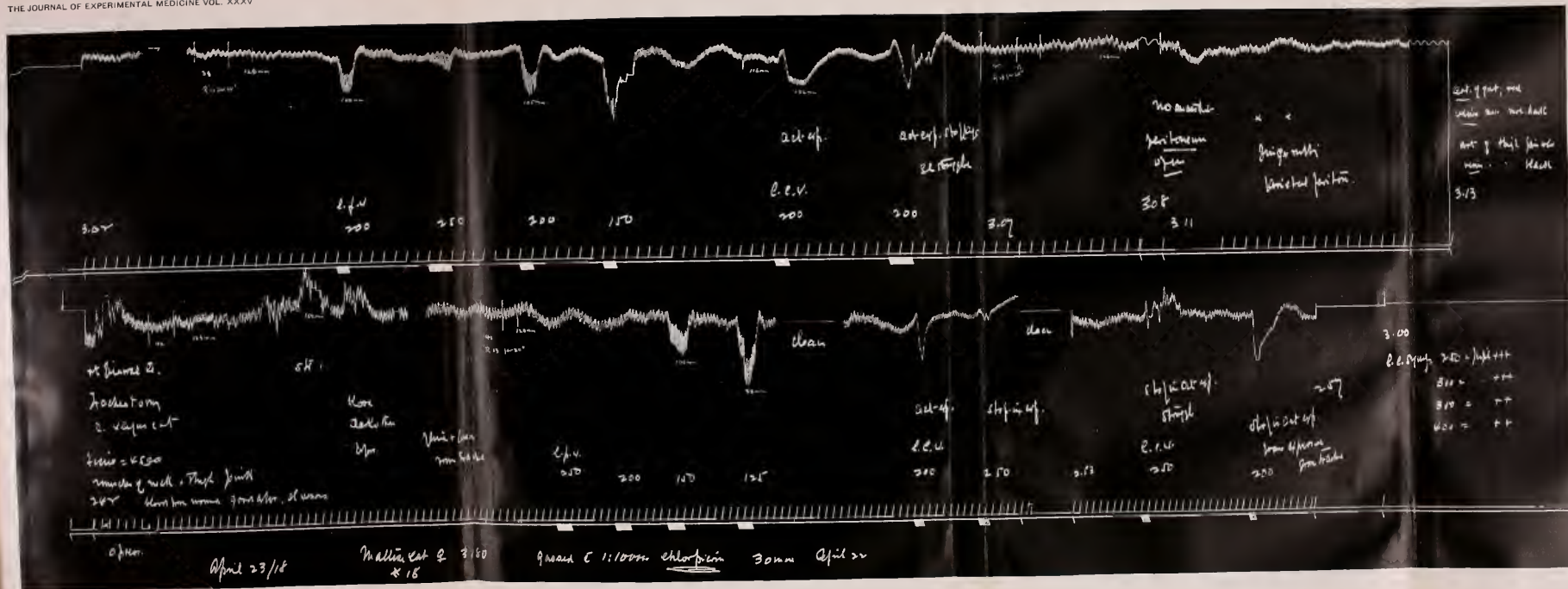


FIG. 1.

(Avert Analgesia after exposure to war gases.)

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(Auer: Analgesia after exposure to war gases.)

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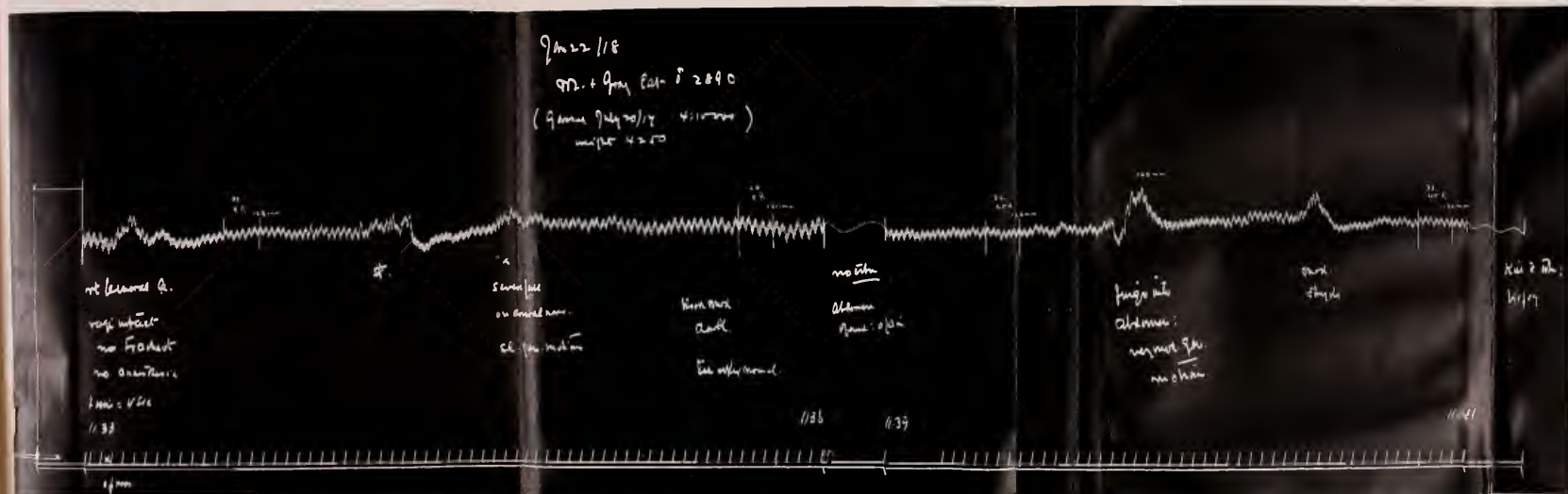


FIG. 3.

(Auer: Analgesia after exposure to war gases.)

EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

II. SURVIVAL OF THE VIRUS IN AEROBIC AND ANAEROBIC CULTURE MEDIA.

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In the first paper of this series¹ it was shown that the typhus virus, in inducing the typical experimental disease in guinea pigs, readily invites invasion in the bodies of these animals of a number of bacteria, which complicate the typhus infection but, on the other hand, have no etiological relationship to the disease.

We thought to continue, in attacking the problem of the etiology of this disease, by means of a deductive method; that is, to put the virus to a variety of experimental tests so as to study the nature of any inciting agent which may reside therein. One of these tests will be described in this paper. The test concerns the survival of the virus in different media, with especial reference to the influence of anaerobic conditions. From the results obtained, we hoped to acquire particular facts which might serve as guides for cultivation experiments.

Method.

The following method was employed. The medium to be tested, under aerobic and anaerobic conditions, was inoculated with the virus present in the blood of a guinea pig reacting to experimental typhus fever. After keeping the mixture of medium and blood containing the virus for varying intervals at 37°C., the blood was removed and injected intraperitoneally into guinea pigs to determine the viability of the virus. Its activity was demonstrable when these guinea pigs developed typical experimental typhus fever, characterized by

¹ Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 525.

(a) the transmissibility of the disease from animal to animal, (b) the presence of characteristic vascular lesions, especially in the brain, (c) the absence of secondary invasions with ordinary bacteria, and (d) the development in recovered animals of immunity to subsequent injections of typhus virus.² The virus was determined to be inactive when guinea pigs injected with it failed to develop either the typical experimental disease, or immunity to further injections of typhus virus.

The details of the methods of procedure were as follows:

Virus.—The virus for the following experiments was obtained from the blood of guinea pigs during the height of their reaction to experimental typhus fever. The blood was secured by cardiac puncture³ with a sterile needle and syringe washed in 50 per cent sodium citrate solution. Three strains of virus, described in another communication,² were employed: human and louse strains originally derived from Poland and a human strain obtained from the blood of a Czechoslovakian immigrant arriving at the Port of New York.

Media.—The media used in the following tests comprised: normal rabbit plasma undiluted; normal horse serum diluted one part to three parts of saline solution; veal infusion 2 per cent peptone broth of a pH of 7.4; Smith-Noguchi tissue, ascitic fluid, sealed medium, prepared in a manner described in another paper.⁴ The latter was modified as follows: in one set of experiments, omission of the tissue; in another, of the petrolatum seal; and finally, human ascitic fluid, undiluted.

Production of Anaerobic Conditions.—Comparative experiments were performed with each medium under aerobic and anaerobic conditions. An anaerobic state was produced by overlaying the medium, immediately after inoculation, with melted petrolatum. It has already been demonstrated^{4,5} that this seal produces an effective anaerobic condition in a few hours in the medium underneath.

Procedure.—4 cc. of guinea pig blood, corresponding to the typhus virus, were inoculated into each of two tubes containing an equal amount of the medium to be tested. The presence of the citrate defibrinated the blood and consequently the blood settled to the bottom of the tube. One tube of the inoculated medium was left unsealed, another was overlaid with melted petrolatum (2.5 cc.). Both tubes were then kept in the same incubator at 37°C. for an indicated period of time. To test the activity of the blood, 3 cc. of the supernatant medium were removed, without disturbing the sedimented blood, and the remaining 5 cc. were

² Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

³ Experiments were performed upon animals under deep chloroform anesthesia.

⁴ Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

⁵ Hall, I. C., *J. Bact.*, 1921, vi, 1.

injected intraperitoneally into a guinea pig. The blood in the aerobic and in the anaerobic medium was injected at the same time, so as to avoid an experimental error. To avoid further error, the experiments were repeated. For similar reasons, guinea pigs which failed to show the typical reaction after injection were observed for a period of about 1 month before they were submitted to an immunity test.

RESULTS.

Virus without Addition of Medium.—As a control to aid in the interpretation of the experiments, 4 cc. of active blood, obtained from guinea pigs during the height of reaction to experimental typhus fever, were kept in a test-tube at 37°C. without the addition of any medium. The blood was kept in the incubator for 24, 48, and 72 hours. At the end of these periods, it was injected intraperitoneally into guinea pigs. Only the blood which was exposed to 37°C. for 24 hours induced typical experimental typhus fever; exposure beyond 24 hours failed to set up either the disease, or immunity to subsequent injections of the virus.

Virus Added to Broth.—Under aerobic conditions, the virus in the blood was active after remaining in this medium for periods ranging up to 5 days. After the 5th day, the virus failed to induce either the experimental disease or immunity.

Under anaerobic conditions, the virus was active after remaining in this medium for 24 and 48 hours, but failed to induce either the disease or immunity after periods longer than 48 hours.

Virus Added to Normal Rabbit Plasma.—Under aerobic conditions, the virus in the blood survived in this medium for 5 days, but failed to induce the disease or immunity in guinea pigs after longer periods.

Under anaerobic conditions, the virus survived for 24 hours, but not thereafter.

Virus Added to Normal Horse Serum.—Under aerobic conditions, the virus in the blood survived in this medium for 5 days but not thereafter.

Under anaerobic conditions, the virus survived for 24 hours, but not for longer periods.

Virus Added to Human Ascitic Fluid Media.—Under aerobic conditions, in human ascitic fluid media with or without the addition of a fragment of fresh rabbit kidney tissue, the typhus virus survived

for periods of 48 and 72 hours at 37°C., but lost its activity after longer periods of exposure.

Under anaerobic conditions, in human ascitic fluid medium without rabbit kidney tissue, the virus remained viable for a period of 48 hours, but not after a longer period. In human ascitic fluid medium, to which was added a fragment of sterile rabbit kidney tissue, and which was then sealed with petrolatum—identical with the anaerobic Smith-Noguchi medium—the virus remained active for only 24 hours. In this medium after 2, 3, 4, and 5 days, the virus failed to induce

TABLE I.

Effect of Different Media, under Aerobic and Anaerobic Conditions, on Typhus Virus.

Medium.	Condition of oxygenation.	Period of survival of virus.
		days
Broth.	Aerobic.	5
	Anaerobic.	2
Normal rabbit plasma.	Aerobic.	5
	Anaerobic.	1
Diluted normal horse serum.	Aerobic.	5
	Anaerobic.	1
Human ascitic fluid (with or without rabbit kidney).	Aerobic.	3
	Anaerobic.	2
Smith-Noguchi medium.	"	1

either the disease or immunity to further injections of typhus virus. The addition of dextrose broth to this anaerobic medium did not prolong the period of viability of the virus.

Table I summarizes these results.

These experiments show that the typhus virus, as it is found in the blood of guinea pigs reacting to the experimental disease, if kept at 37°C. in different media from which oxygen is excluded by a petrolatum seal, tends to die off rapidly, in 24 to 48 hours. On the other hand, the period of survival is prolonged to 5 days (in only one case to 3 days) when the same media have free access to atmospheric oxygen.

DISCUSSION AND SUMMARY.

The results of the foregoing experiments show that the typhus virus, found in the blood of guinea pigs during the height of typical experimental typhus fever, does not survive at 37°C. in anaerobic media for as long a period as in the same media under aerobic conditions. In media from which oxygen is excluded, the viability period is 24 to 48 hours; in the same media having no barrier to atmospheric oxygen, the period is usually 5 days, in one instance, 3 days.

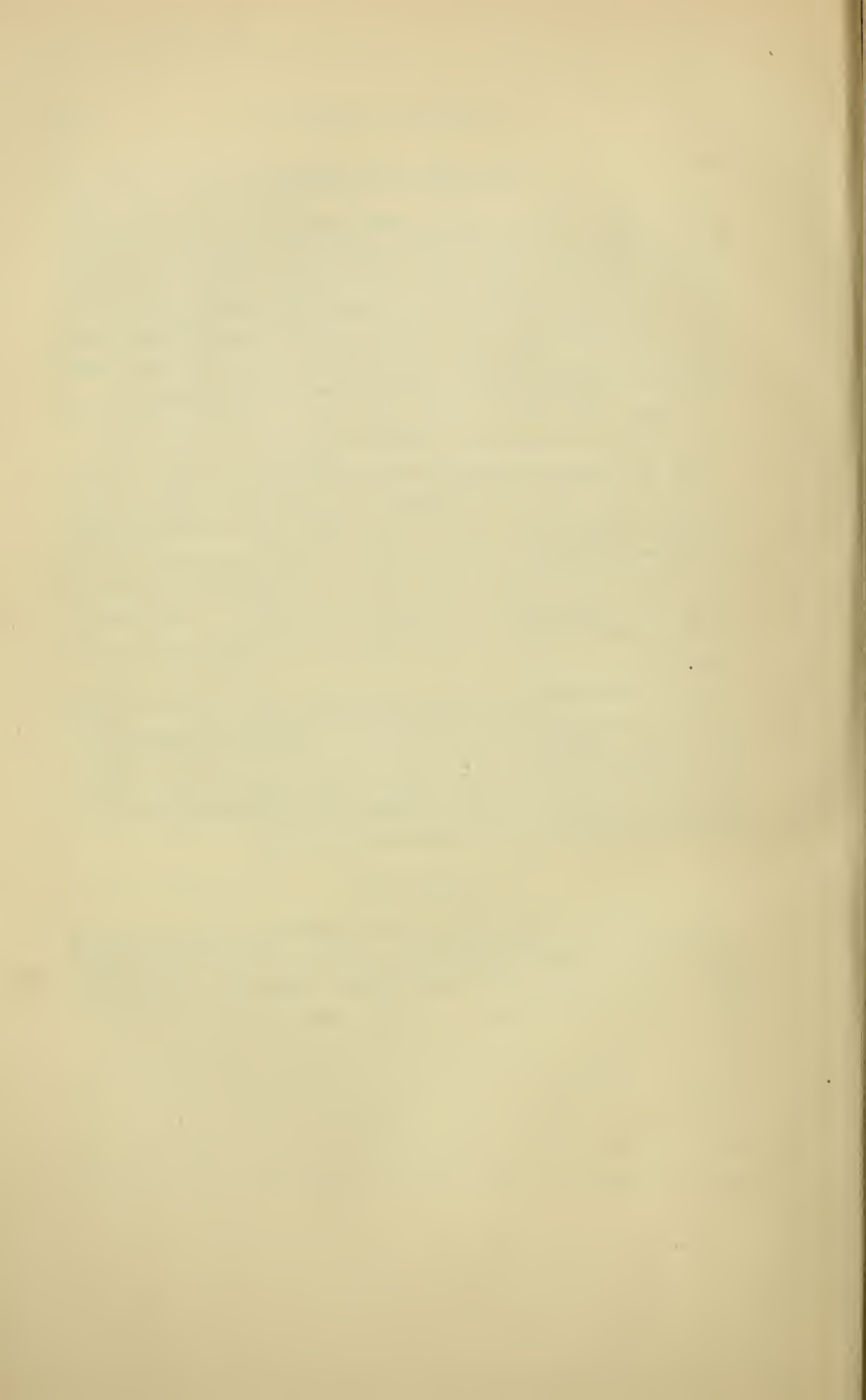
The dead virus fails to induce not only the typical experimental disease but also an immunity to further injections of typhus virus.

That the death of the virus is due to exclusion of oxygen from the medium, rather than to a change in the hydrogen ion concentration therein is inferred from the fact that media with varying hydrogen ion concentrations, such as broth (pH 7.4), horse serum (pH 7.8), and human ascitic fluid (pH 8.0) have the same comparative effect on the virus, when under aerobic and anaerobic conditions. That is, in all, the anaerobic state causes a shortening of the viability period of the typhus virus.

In the Smith-Noguchi tissue, ascitic fluid, sealed medium in which bacteria resembling Plotz' bacilli grow luxuriantly and remain viable for several weeks, the typhus virus does not increase in virulence, and even dies after 24 hours. This evidence supports the conclusion previously presented¹ that the *Bacillus typhi exanthematici* of Plotz is not identical with the active agent of typhus virus.

CONCLUSION.

In a variety of media kept at 37°C., and from which oxygen is excluded by a petrolatum seal, the typhus virus tends to die rapidly. In the corresponding media under aerobic conditions, the life of the virus is appreciably longer.



EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

III. FILTRATION EXPERIMENTS.

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In a previous paper¹ we presented a plan of studying the problem of the inciting agent of typhus fever by the deductive method; that is, by applying to the typhus virus a variety of known tests in order to determine the nature of the particular incitant which may reside therein. From the experiments performed, it was concluded that the virus, in inducing experimental typhus fever in guinea pigs, invites invasion of the body of the animals by a variety of bacteria, which complicate the infection but have no etiological relationship to typhus,² and that the virus itself survives for a longer period in aerobic than in anaerobic culture media.¹

In this article, the deductive method is continued and additional experiments on the typhus virus are presented relating to the filterability of the virus as it exists in the organs of guinea pigs during the height of the reaction to inoculation.

HISTORICAL.

Heretofore, most of the experiments on the filterability of typhus virus have been made with the blood taken from guinea pigs or monkeys at the height of the experimental typhus infection. Goldberger and Anderson,³ after reviewing all previous experiments, state that there have been eight attempts, by different workers, to pass the virus of typhus through a Berkefeld filter. Of these, six were negative; in one of the other two (Wilder, 1911⁴), the monkey that had been

¹ Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 115.

² Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 525.

³ Goldberger, J., and Anderson, J. F., *Bull. Hyg. Lab., U. S. P. H.*, No. 86, 1912, 62.

⁴ Wilder, R. M., *J. Infect. Dis.*, 1911, ix, 9.

inoculated with the filtrate, without giving any evidence of infection, was later found to be resistant to an immunity test with active blood; in the other (Nicolle, Conor, and Conseil, 1910⁵), one of two monkeys inoculated with filtrate is described as having presented a doubtful reaction, and later was found to be resistant to an inoculation with active blood.⁶ Goldberger and Anderson then described their own experiments, which convinced them that the virus, as it exists in the blood, is not filterable.

Later, in 1914, Nicolle, Blanc, and Conseil⁷ inoculated into monkeys filtrates prepared from lice harboring the virus, and while no typical reaction resulted, it was found that on reinjection with active blood only a delayed and mild reaction occurred, which these workers regarded as evidence of a partial immunity.

In 1917, the writer's⁸ efforts to filter the virus in the blood of experimentally infected guinea pigs during the reaction were fruitless. He concluded that the virus was not filterable.

The foregoing review shows that as yet no conclusive experimental basis has been offered to prove that in typhus blood the virus is filterable, although Nicolle and his associates hold that opinion.

In 1911, Nicolle, Conor, and Conseil⁹ assumed that the virus of typhus fever is an intracellular parasite. Later observers, especially those who have linked the *Rickettsia prowazeki* with the incitant of typhus fever (da Rocha-Lima,¹⁰ Wolbach and Todd,¹¹ and others) have taken the same stand.

The experiments to be described relate to two points: first, the supposed intracellular nature of the inciting agent, and second, the filterability of the virus. In order to approach more nearly ideal conditions of experiment, we employed organs derived from the infected animals instead of the blood.

Methods.

The spleen and brain of typhus-infected guinea pigs at the height of their reaction were chosen. Two strains of virus from human

⁵ Nicolle, C., Conor, A., and Conseil, E., *Compt. rend. Acad.*, 1910, cli, 685.

⁶ In a later communication, we shall present evidence to show that the production of immunity in isolated instances after the injection of filtrates may possibly be dependent on other factors than a living multiplying agent.

⁷ Nicolle, C., Blanc, G., and Conseil, E., *Arch. Inst. Pasteur Tunis*, 1914, ix, 84.

⁸ Olitsky, P. K., *J. Infect. Dis.*, 1917, xx, 349.

⁹ Nicolle, C., Conor, A., and Conseil, E., *Compt. rend. Acad.*, 1911, cliii, 578.

¹⁰ da Rocha-Lima, H., *Arch. Schiffs- u. Tropen-Hyg.*, 1916, xx, 17.

¹¹ Wolbach, S. B., and Todd, J. L., *Ann. Inst. Pasteur*, 1920, xxxiv, 153.

sources were used: one originating at Warsaw,¹² the other obtained from a Czecho-Slovakian immigrant at the Port of New York. Both strains have been described in a previous report.¹³ That the spleen and brain are capable of inducing the experimental disease in guinea pigs has been shown by Nicolle and Blaizot¹⁴ as well as by later workers. Indeed, Landsteiner and Hausmann¹⁵ have induced experimental typhus fever in guinea pigs by inoculating as small a dose as 0.005 gm. of the infected brain tissue.

The first experiments refer to the problem of the supposed intracellular location of the virus and to an attempt to free it through cellular disintegration.

Repeated Freezing and Thawing.—An infected guinea pig, at the height of its reaction, was exsanguinated.¹⁶ The spleen was removed under sterile conditions; one-fifth was kept for a control test and the remainder finely pulped with scissors. The pulp was placed in a test-tube containing 20 cc. of Ringer's solution, and frozen solid by means of calcium chloride in cracked ice at -26°C . The frozen mass was then thawed out to the original fluid condition and immediately refrozen. A small portion of the pulp was removed and tested for growth *in vitro* with negative results.¹⁷ In the meanwhile, the remainder of the pulp in suspension, with the exception of 2 cc. employed in the control test, was filtered through a tested Berkefeld V or N candle and the filtrate inoculated in the manner to be described.

Freezing and Desiccating.—In this test the spleen was frozen and then desiccated to a flaky powder according to the method of Swift.¹⁸ As in the case of the previous method, controls of tissue before desiccation were employed. The remaining four-fifths of the desiccated splenic tissue was suspended in Ringer's solution and filtered after 4 cc. had been removed for control purposes.

Crushing in a Mechanical Tissue Crusher.—The same conditions existed in this test as in the above, except that the spleen was placed in a mechanical tissue crusher and the tissue juice and very fine homogeneous semifluid pulp resulting were suspended in Ringer's solution and filtered, the 2 cc. of the original unfiltered material being withheld for control purposes.

¹² We owe this strain to the kindness of Dr. S. B. Wolbach.

¹³ Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

¹⁴ Nicolle, C., and Blaizot, L., *Arch. Inst. Pasteur Tunis*, 1916, ix, 127.

¹⁵ Landsteiner, K., and Hausmann, W., *Med. Klin.*, 1918, xiv, 515.

¹⁶ All operations were performed under deep chloroform anesthesia.

¹⁷ This test was performed by Dr. Alexis Carrel.

¹⁸ Swift, H. F., *J. Exp. Med.*, 1921, xxxiii, 69.

Grinding with Sand.—In the same way, the cut up fragments of spleen were ground with sterile fine white sand in a mortar, with the addition, from time to time, of 5 cc. of Ringer's solution until the required amount (20 cc.) was obtained. All but 2 cc. of the thin, even, homogeneous suspension was then filtered.

Precisely similar procedures were carried out with brain tissue from infected guinea pigs.

Of the four methods employed in treating the cellular elements of the tissues, those of repeated freezing and thawing at $-26^{\circ}\text{C}.$, and freezing and desiccating are known to kill the cells completely.¹⁹ Crushing or grinding tissues destroys the cells, but it is presumed that an occasional living cell may escape. However, the following experiments demonstrate that the virus in the tissues prepared by any of these methods retains an undiminished infecting power. Hence one method serves to control the others.

There were thus available for testing (a) typhus-infected tissues of guinea pig spleen and brain, (b) the same tissues, either frozen, desiccated, crushed, or ground, and suspended in Ringer's solution, and (c) filtrates of these suspensions which were freed from the tissue cells and detritus.

Procedure.—Each experiment comprised six animals. Guinea Pig A was injected intraperitoneally with 2 cc. of the suspension of infected tissue. This animal served as a control to test the infecting power of the virus in the tissue.

Guinea Pig B was injected intraperitoneally with 2 cc. of the suspension of the unfiltered suspensions of infected tissue, either frozen, desiccated, crushed, or ground, in the order of the tests made. This animal served as a control to test the effect on the virus of the methods used in disintegrating the tissue cells.

Guinea Pigs C and D were injected with 5 cc. of the filtrate of the particular suspension of the tissue (either frozen, desiccated, crushed, or ground) to be tested. Of these animals, Guinea Pig C was allowed to run its course so as to detect any delayed reaction and, in certain instances, was reinjected, after a suitable interval, with active typhus virus in order to test its immunity. Guinea Pig D was killed from 4 to 7 days after injection,²⁰ and 3 cc. of its defibrinated blood were inoculated into each of two normal animals, E and F. In this way the possibility of transmission of a living, multiplying agent might be detected and the pathological changes in the body of Guinea Pig D studied.

¹⁹ For the literature on this subject, see Wells, H. G., *Chemical pathology*, Philadelphia, 4th edition, 1920, 373.

²⁰ During this period the blood of guinea pigs experimentally infected contains active virus.²

RESULTS.

In all, fourteen experiments, as detailed above, were made with infected spleen and brain derived from guinea pigs at the height of the experimental infection: Three were made with the frozen infected tissue; two with the desiccated tissue; three with the crushed tissue, and six with the ground tissue. The results of the different experiments were practically identical.

Guinea pigs of Series A, injected with the infected tissue which was not disintegrated, showed, after an incubation of from 5 to 10 days, typical experimental typhus fever. On transmission by means of the blood obtained during the height of the reaction to normal animals, the latter, in turn, exhibited the typical fever. This was characterized by the specific histopathological picture, immunity to subsequent injections of active virus, freedom from concurrent or secondary infections, and transmissibility of the fever to normal animals—thus agreeing with the definition of the experimental disease described elsewhere.¹³ Hence the materials employed for the filtration tests were proved to have specific infecting power.

Guinea pigs of Series B, injected with the infected tissue which had been either frozen, desiccated, crushed, or ground, exhibited the typical experimental typhus fever paralleling that shown by the animals of Series A.²¹ Therefore, the tissue cells killed by repeated freezing and thawing, or by freezing and desiccating, or crushed into detritus by mechanical means, or ground into a homogeneous fine pulp, have had no effect on the infecting power of the typhus virus.

Guinea pigs of Series C and D, inoculated with the filtrates of suspensions of the frozen, desiccated, crushed, or ground infected tissue, failed to show the typical experimental disease. In five experiments, the animals of Series C were reinjected with active typhus virus; all failed to show any immunity. In ten experiments, the blood of guinea pigs of Series D, obtained 4 to 7 days after injection with the filtrates, was inoculated into normal animals (Series E and F). In none of these was there induced either typical experimental typhus

²¹ The unfiltered desiccated tissues were injected 6 days after drying; in the instances in which the tissues were disintegrated by the other methods, the injections were made immediately after disintegration.

fever or immunity. Hence the filtrates failed both to cause the typical disease and to produce immunity.

To summarize the results of these experiments, we find that fourteen attempts to filter the active typhus virus present in the brain or spleen of guinea pigs at the height of reaction have ended in failure.

SUMMARY AND CONCLUSIONS.

We have presented experiments to show that the typhus virus in the tissues of the guinea pig during the height of reaction to the experimental disease does not lose its infecting power when the cells of the brain or of the spleen are disintegrated by repeated freezing and thawing, or by freezing and desiccating, or by crushing by mechanical means, or by grinding into a homogeneous pulp with sand. The virus after such treatment is as actively infective as in the same tissue not subjected to the disintegrating influences. The possibility exists, therefore, of an extracellular condition of the typhus virus.

Fourteen attempts to filter through Berkefeld V and N candles the virus contained in the disintegrated tissue have all resulted in failure.

DO SPECIES LACKING A GALL BLADDER POSSESS ITS FUNCTIONAL EQUIVALENT?

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It is a singular fact that certain closely related species of animals have, some of them, a gall bladder, while others do not. What can be the essential significance of the organ and the reason for this difference?

Embryologically, the vesica fellea arises secondarily as a cul-de-sac from the same anlage that forms the liver and bile ducts, and it is considered by many as an almost purposeless diverticulum. The erroneous nature of this view is sufficiently shown by the several functions now demonstrated for the organ. The matter has been discussed in a previous paper from this laboratory.¹ The fact that the gall bladder is a highly specialized organ renders all the more noteworthy its irregular distribution in both high and low forms of life. Thus among the higher animals it is present in the cow and sheep, while it is absent in the horse, present in the goat, and absent in the closely related deer—to be found in the hog and wild boar but not in the peccary of South America. Among birds, the hawk and owl possess it, while doves do not; and among the rodents the mouse is found with the organ, the rat without. One species of gopher,² the pocket gopher (*Geomys bursarius*), is without a gall bladder, while another (*Spermophilus tredecimlineatus*) the striped gopher, possesses it. Woods Hutchinson³ is authority for the statement that in the giraffe it is at times present and again not.

¹ Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

² Mann, F. C., *J. Lab. and Clin. Med.*, 1919-20, v, 107.

³ Hutchinson, Woods, *Med. Rec.*, 1903, lxiii, 770.

The disposition of the bile ducts or the position of the pancreas throws no light on the riddle of the gall bladder. If we consider only animals without the organ, the horse has separate pancreatic and common bile ducts emptying into the duodenum through a common opening. The deer, rat, and pocket gopher (*Geomys bursarius*) show the pancreatic duct opening directly into the common bile duct well above the ampulla, this it also does in both rats and white mice, the former being without a gall bladder, the latter with it. No inference can be drawn from the high opening of the pancreatic duct, for this is as inconstant⁴ as the presence of the gall bladder itself.

The vesica fellea has, as already indicated, functions sharply different from those of the ducts. Its rôle as a reservoir may on occasion, as after cholecystectomy, be partially taken over by the ducts.⁵ Not so, however, with its modifying influences upon the bile. The bladder acts to concentrate the secretion greatly and to thicken it with mucus, whereas the ducts by contrast tend to dilute it, though to a negligible degree, with a thin product of their own.⁶ Is it possible that in animals lacking the gall bladder, the concentrating function is lodged somewhere in the duct wall? This possibility can readily be tested out by ligation experiments on rats and mice.

If the gall bladder has the same activities in the mouse as in the dog, cat, and monkey,¹ then the bile submitted to its influence after ligation of the common duct should become concentrated by the action of the gall bladder. Granting that this really occurs,—and the findings now to be described show that it does,—will a concentration of bile also go on in the obstructed duct system of the rat, an animal devoid of a gall bladder, or will this bile be diluted as is the case with bile pent in the ducts of other animals which have been deprived of their connection with the bladder? The close relationship of the species and their similarity in habits give a special value to the test.

⁴ Mann, F. C., Foster, J. P., and Brimhall, S. D., *J. Lab. and Clin. Med.*, 1919–20, v, 203.

⁵ Haberer, H., and Clairmont, P., *Verhandl. deutsch. Ges. Chir.*, 1904, xxxiii, pt. 2, 81. Rost, F., *Mitt. Grenzgeb. Med. u. Chir.*, 1913, xxvi, 710.

⁶ Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 75.

Method.

The pigment content of the bile was taken as the criterion of extra-hepatic changes in concentration. Its utility in this connection has already been shown.¹ The range of the normal pigment content of mouse and rat bile was first determined with the animals on their ordinary ration (bread and milk, and grain).

From rats the bile was obtained into small rubber bags connected with fine cannulas inserted in the common duct. The bags were left within the animal's peritoneal cavity for 24 hours or more. Considerable amounts of secretion, ample for pigment analyses, were readily obtained in this way. The small size of the mouse, however, made necessary a different method, which will later be described.

All operations were carried out under ether, with aseptic precautions. To produce stasis, a triple ligation of the duct was done, the ligatures being placed just below the junction of the lowest hepatic duct with the common duct and well above the pancreatic duct entrance, and the common duct was then cut between the middle and lowest ties. The animals were allowed to live thereafter for periods of 1 to 16 days on a mixed diet of barley and oats, with bread and milk. Recovery from the operative procedure was rapid and the animals ate well. They were killed at intervals, the stasis bile was collected into a pipette, and its pigment content determined. In no instance had the continuity of the duct been reestablished. Cultures of the bile and liver tissue were regularly taken.

The Quantitation of Pigment.

Different methods were used to determine the pigment content of bile according to the amount available. When more than 0.3 or 0.5 cc. of rat bile was had, a reading was carried out according to the colorimetric method of Hooper and Whipple.⁷ To a measured quantity of bile is added a known quantity of acid alcohol reagent. For a standard, instead of a wedge of artificial constitution, a chloroform solution of pure bilirubin (Schuchardt) was used, which was mixed with the reagent, as was the bile, and read against it in a Duboscq colorimeter.

When the quantity of rat bile was very small, the mixture of it with the acid alcohol reagent was made in a Miescher pipette such as is used in the Fleischl-Miescher hemoglobinometer and read against the standard bilirubin solution as ordinarily, but in a micro colorimeter.

⁷ Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

The pigment content of normal mouse bile in the small quantities obtained was always insufficient to yield, with the reagent, a green color that could be read in the colorimeter. It was quantitated directly against a potassium bichromate scale, a method used as well with rat biles. For the purpose, glass tubes with a bore of 0.75 mm. and walls so thick (2 mm.) as to result in a magnification, were filled with bile and with the scale solutions. The tint of bile and standard matched very closely. A permanent series of tubes was made containing dilutions of potassium bichromate ranging from 6.0 to 0.05 per cent; and tests were made to determine whether the color intensity of mouse bile varied on dilution like that of a weak bichromate solution. Specimens of bile which corresponded in color with bichromate solutions of known percentage were diluted and compared again with the scale. They now agreed with a percentage bichromate solution corresponding to their dilution. Thus a bile solution corresponding in color with a 1 per cent bichromate solution, when diluted with equal parts of water, had the color strength of a 0.5 per cent bichromate solution—when with ten parts of water it corresponded with a 0.1 per cent solution, and so on. In order to standardize the scale, a number of normal rat biles of known pigment content, taken at random, were compared with the bichromate tubes. The results of this comparison have been assembled in Table I. There three subdivisions have been made according to the pigment strength of the bile employed. The bilirubin content in milligrams per 100 cc., as determined by the acid alcohol reagent, is given in Column A, and its reading in percentage of bichromate solution, as determined by comparison with the small glass tubes, is given in Column B. If the relative amount of pigment can be read as truly on the bichromate scale as by the Hooper and Whipple method, then $\frac{A}{B}$ should yield a constant, which constant incidentally is the expression in milligrams of bilirubin per 100 cc. of bile, of the colorimetric value of a 0.1 per cent bichromate solution, or for a 1 per cent solution, when multiplied by 10. It will be seen that the constant was only approximately yielded by the individual bile specimens, but the variation is much the same throughout the groups, and the average for the constant was 13.26, 13.51, and 13.57 for biles of

TABLE I.
Standardization of the Potassium Bichromate Scale.

Rat No.	A Actual amount of bilirubin per 100 cc.	B Per cent strength of bichromate solution correspond- ing in color to the bile.	$\frac{A}{B} \times 10$	Averages.
	mg.			
8	8.9	0.7	12.7	Pigment less than 15 mg. per 100 cc. of bile. Average 13.26
37	11.7	0.8	14.6	
38	12.5	0.9	13.77	
9	14.38	1.2	11.98	
7	15.43	1.4	11.02	Pigment amounts 15 to 20 mg. per 100 cc. of bile. Average 13.51
6	16.8	1.4	12.0	
14	17.85	1.2	14.88	
15	19.1	1.2	15.91	
10	19.23	1.4	13.75	
5	20.16	1.8	11.2	Pigment amounts above 20 mg. per 100 cc. of bile. Average 13.57
16	20.83	1.5	13.88	
13	22.32	1.6	13.9	
11	22.72	1.5	15.15	
12	24.51	1.8	13.6	

All bile collections were made by the bag-cannula method.

Rat biles collected by the bag-cannula method were employed. In Column A, the actual milligrams of pigment per 100 cc. of bile, as obtained by the acid alcohol method, are given. Column B gives the pigment strength of the bile in per cent of potassium bichromate solution as obtained by direct color comparison.

If this latter comparison is a true one, then $\frac{A}{B}$ should yield an expression in milligrams of bilirubin per 100 cc. of bile of the colorimetric value of a 0.1 per cent bichromate solution, as determined from that particular specimen of rat bile.

This multiplied by 10 should give the figure for a 1 per cent solution ($\frac{A}{B} \times 10$), and should be a constant. The readings are separated into three groups according to whether the pigment content of the bile fell below 15 mg. per 100 cc., between 15 and 20 mg., and over 20 mg. per 100 cc. It will be seen that the same approximate constant ($\frac{A}{B} \times 10$) is yielded by all three groups.

low, medium, and high pigment content respectively, the general average being 13.45. As the table shows, a bile which reads on the bichromate scale at 1 per cent will contain about 13.45 mg. per 100 cc. Thus a specimen of rat or mouse bile obtained from a duct could be placed in one of the small bore tubes, compared with the bichromate scale, and its pigment content quantitatively estimated.

The Pigment in Normal Mouse Bile.

The delicacy of the common duct in mice prohibited the use of the bag and cannula method which was successful with rats. Instead, bile was collected from animals under ether, through extremely fine long cannulas, with the aid of suction.

To obtain cannulas of sufficient flexibility, hard glass tubing was drawn out into capillary pipettes with an external diameter of about $\frac{1}{3}$ mm. and a length of 40 to 60 cm., with the small end carefully rounded to avoid laceration of the duct. The larger end of the tube was curved and the bend in it formed a sort of basin into which the bile from the capillary collected. Mild suction was maintained at this end, when desired, through a connection with a rubber tube and bulb filled with water, which could be lowered at will.

The larger end of the cannula was rigidly attached to a mechanical stage which permitted accurate movement in two dimensions of space and its flexible shaft was passed through three fixed wire loops, so arranged as to give it a marked downward curvature. All of the loops could be raised or lowered at will, thus increasing or decreasing the curvature of the shaft and correspondingly deflecting the open end of the cannula, so that it could be made to take the exact direction of the bile duct in the individual mouse. This adjustment was essential to prevent kinking and obstruction of the duct.

Under ether, the bile duct was exposed close to its entrance into the duodenum. By the use of the mechanical stage the cannula was then introduced either through a slit in the duct or by ligating the duodenum to either side of the papilla of Vater, opening it and thrusting the cannula through the papilla into the duct. A magnifying glass placed above the field of operation was found of service. The cannula once in the duct was always advanced to a point well above the entrance of the channel and in many instances tied in place, thus blocking off all pancreatic secretion. Often it plugged the duct so tightly that this was unnecessary. Mild suction was produced on the capillary pipette and the animal kept under ether for several hours while the flow of bile went on.

By this arrangement the gall bladder was left in direct connection with the cannula and *a priori* one would suppose that its contents

formed part of the normal bile collected through this latter. However, on first opening the abdomen to insert the cannula, the gall bladder was usually noted to be empty. Even when full, little of its contents was yielded to the cannula on suction.

Collections were successfully made by this method for periods as long as $6\frac{1}{2}$ hours. Body warmth was kept up by electric lights placed near the animal, and injury was combated with moist sponges. The bile obtained was very uniform in character, exceedingly abundant, clear, light amber-yellow but, as already said, too weak in pigment, in the small quantities obtainable, to yield a notable blue-green color with the acid alcohol reagent.

Table II shows the result of readings against the bichromate scale with the estimated amounts of bilirubin per 100 cc. of bile. An average from ten cases gives a pigment content of 2.17 mg. of bilirubin per 100 cc. of bile. The quantity of bile secreted per 100 gm. of body weight in an hour was about 0.2 cc., or, per kilo of body weight per day (24 hours), 34.0 cc. A gram of liver tissue of the mouse secreted about 0.03 cc. of bile per hour.

Results of Obstruction in Mice.

The bile was studied from many mice in which obstruction had been produced by ligation of the common duct. Obstruction lasting 1 to 3 days did not result in a marked dilatation of the ducts but the gall bladder was always found full of a bile much darker and more mucinous than normal mouse bile. It had early a clear, dark amber color, and the longer the obstruction the darker the bile. After 3 days, the ducts became dilated, though the gall bladder did not greatly increase in size. Both now contained abnormally dark bile in greater and greater amount from day to day. The actual quantity was difficult to measure, since in puncturing the ducts some was almost always lost.

In accordance with expectations based on the findings in larger animals with gall bladders,¹ the stasis bile always contained much more than the normal amount of bilirubin (Table III). When stasis had existed for more than 24 hours, the gall bladder bile had sometimes a dark greenish tint. This change of some of the pigment to biliverdin caused difficulty in the comparison with the bichromate

scale. Fortunately, the pigment amount was so great as to yield, even in the small quantities of bile at hand, the acid alcohol reaction of Hooper and Whipple whereby biliverdin and bilirubin are quantitated together.

Table III shows that the bile obtained after 1 day of stasis ranged in color strength, as expressed in terms of the bichromate scale, from 1.5 per cent bichromate to 4 per cent, with an average of 2.25 per

TABLE II.
Pigment Content of Normal Mouse Biles.

Mouse No.	Mouse weight.	Liver weight.	Period of collection.	Total bile.	Quantity of bile per 100 gm. of body weight per hr.	Quantity of bile estimated per kilo of body weight per day.	Quantity of bile per gm. of liver per hr.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimated from reading of bichromate scale.
	gm.	gm.		cc.	cc.	cc.	cc.		mg.
1	23.0	1.15	2 hrs., 45 min.	0.1+	0.158	38.0	0.032	—	—
2	21.6	1.4	4 " 15 "	0.06 (incomplete).	0.065	15.6	0.011	0.1	1.35
3	20.0	1.4	1 hr., 25 "	0.15	0.53	12.7	0.075	0.1	1.35
4	24.5	1.9	4 hrs., 20 "	0.29	0.273	65.5	0.035	0.1	1.35
5	23.3	1.2	5 " 5 "	0.14	0.118	28.3	0.023	0.15	2.02
6	24.0	1.2	2 " 50 "	0.04	0.059	14.2	0.012	0.3	4.03
7	27.0	1.9	2 " 50 "	0.12	0.157	37.6	0.022	0.2	2.69
8	24.0	1.8	5 " 20 "	0.32	0.25	60.0	0.033	0.1	1.35
9	—	—	5 " 40 "	0.18	—	—	—	0.25	3.36
10	—	—	3 " 10 "	0.05 (incomplete).	—	—	—	0.15	2.02
Average.....					0.20	34.0	0.03	0.16	2.17

cent. In normal animals, by contrast, (Table II) the range was from 0.1 to 0.3, and averaged 0.16 per cent. The stasis bile, then, was fourteen times as strong in pigment as the normal. After 1 day of stasis, no perceptible conversion of bilirubin to biliverdin had occurred.

In a number of cases bile found in the gall bladder was compared with that collected from the ducts of the same animal. The pigment strength of the bladder and duct biles were compared with the aid of the bichromate scale (Table IV).

TABLE III.
Pigment Content of Mouse Biles from Obstructed Ducts.

Mouse No.	Mouse weight.	Liver weight.	Period of obstruction.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimated from reading of bichromate scale.	Actual amount of bilirubin per 100 cc. of bile (acid alcohol method).
	gm.	gm.	days		mg.	mg.
11	27.8	2.25	1	2.0	26.89	—
12	23.0	1.7	1	4.0	53.78	52.08
13	19.5	1.2	1	2.0	26.89	—
14	24.2	2.2	1	1.5	20.17	—
15	21.5	1.35	1	2.2	29.58	26.04
16	27.75	1.55	1	—	—	82.6
17	—	—	1	—	—	47.7
18	25.4	2.1	1	2.0	26.89	27.77
19	25.5	1.85	2	—	—	—
20	23.0	1.2	2	—	—	52.1
21	25.0	1.9	2	—	—	38.1
22	—	—	13	—	—	32.0
23	—	—	14	—	—	35.0
Average.....				2.25 (after 1 day of obstruction only).		

Average pigment content of bile after 1 day of obstruction is 30.7 mg. per 100 cc.

TABLE IV.
Comparison of the Pigment Strength of the Bladder and Duct Biles from Mice at the Close of the Bile Collection.

Mouse No.	Results.											
4	Gall bladder bile showed 7 times the concentration of duct bile after a 4 hrs. collection.											
5	"	"	"	"	4	"	"	"	"	"	"	5
6	"	"	"	"	3	"	"	"	"	"	"	3
7	"	"	"	"	5	"	"	"	"	"	"	3
8	"	"	"	"	5	"	"	"	"	"	"	5 $\frac{1}{3}$
9	"	"	"	"	4	"	"	"	"	"	"	5 $\frac{3}{4}$
Average.	"	"	"	"	4.66	"	"	"	"	"	"	4.3

Normal and Stasis Biles of the Rat.

The rubber bags used to obtain normal bile from rats were made out of finger cots and attached to glass cannulas of capillary diameter, with the ends well rounded and the shaft curved to avoid torsion on the duct. The cannula was fastened in the duct with ligatures above the entrance of the pancreatic duct, and in some instances the pancreatic duct as well was tied. Such fat necrosis as sometimes followed this latter procedure seemed not to affect the animal materially in the short period of bile collection. The rats ate well after the operation. At the end of 18 to 24 hours they were killed with chloroform.

To produce obstruction, ligature and severance of the common duct was employed. Bile leaks into the peritoneal cavity seldom were found and animals in which they occurred were discarded. Tissue icterus always appeared in 24 hours and bile pigment was plentiful in the urine. The twenty operated animals were killed at intervals ranging from 1 to 16 days after obstruction.

At autopsy the degree of dilation of the obstructed bile ducts was noted and the approximate amount of bile present within them. Very occasionally positive cultures were obtained from bile or liver tissue of the rats into which bags had been inserted, but, probably owing to the short period of collection, such biles seemed otherwise normal. The not infrequent infected cases among the animals with obstruction were ruled out. The collecting bags gave an abundant yield of bile—never less than 1.5 cc. and as much as 6.5 cc. per 100 gm. of body weight in 24 hours.

From Table V it will be seen that the pigment content of normal rat bile as determined in sixteen animals was 17.05 mg. of bilirubin per 100 cc., with a variation of 8.9 to 22.3 mg. It is of interest to note that the bulk of bile secreted per gram of liver tissue per hour in rats and mice is almost identical, but the proportion of liver weight to body weight in rats, 1 to 21.7, is considerably smaller than that of mice, 1 to 14.6. The quantity of bile secreted per 100 gm. of body weight was, per hour, 0.151 cc., or per kilo of body weight per day (24 hours) 36.3 cc.; and of bile per gram of liver per hour 0.034 cc., or per day 0.82 cc.

The normal rat bile was always a clear amber fluid as viewed in a test-tube, quite transparent, and a brilliant light yellow when placed in one of the reading tubes for comparison with the bichromatic scale. It showed remarkably little variation in pigment concentration, but had as a rule relatively less color when the amount of bile

TABLE V.
Normal and Stasis Biles of the Rat.

Pigment content of normal biles.										Pigment content of biles from obstructed ducts.							
Rat No.	Rat weight. gm.	Liver weight. gm.	Period of collection. hrs.	Total bile. cc.	Quantity of bile per 100 gm. of body weight per hr.	Quantity of bile esti- mated per kilo of body weight per day. cc.	Quantity of bile per gm. of liver per hr. cc.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimated from reading of bi- chromate scale. mg.	Actual amount of bili- rubin per 100 cc. of bile (acid alcohol method). mg.	Rat No.	Rat weight. gm.	Liver weight. gm.	Period of obstruction. days	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimat d from reading of bi- chromate scale. mg.	Actual amount of bili- rubin per 100 cc. of bile (acid alcohol method). mg.
1	166	8.7	25	3.6	0.087	20.8	0.017	—	—	13.87	17	109.0	6.0	1	1.3	17.48	—
2	158	7.5	25	5.7	0.144	34.5	0.030	—	—	12.33	18	99.0	5.0	1	1.2	16.14	—
3	122	7.0	22½	7.1	0.258	62.0	0.045	—	—	13.55	19	88.7	4.4	1	1.6	21.51	—
4	160	9.5	22	6.2	0.176	42.6	0.030	—	—	13.15	20	92.5	4.2	1	1.4	18.82	—
5	—	—	19	2.4	—	—	—	1.8	24.20	20.16	21	76.5	3.7	2	1.2	16.14	—
6	92	5.8	—	—	—	—	—	1.4	18.82	16.8	22	77.5	5.5	2	1.3	17.8	—
7	178	7.2	18	2.5	0.078	18.7	0.019	1.4	18.82	15.43	23	92.2	5.5	3	1.2	16.14	—
8	163	7.7	22	7.8	0.218	52.2	0.046	0.7	9.41	8.93	24	80.8	5.1	3	1.3	17.8	—
9	158	6.1	22	—	—	—	—	1.2	16.13	11.98	25	—	—	4	1.8	24.2	—
10	163	6.5	18	4.8	0.164	39.4	0.041	1.4	18.82	19.23	26	93.2	4.7	5	1.8	24.2	25.75
11.	170	6.6	18	2.9	0.095	22.8	0.024	1.5	20.17	22.72	27	—	—	5	—	—	14.0
12	158	5.8	18	1.8	0.063	15.1	0.017	1.8	24.20	24.51	28	—	—	5	—	—	15.4
13	153	6.5	18	3.1	0.113	27.1	0.027	1.6	21.51	22.32	29	—	—	5	—	—	10.68
14	107	4.2	18	5.2	0.271	64.9	0.068	1.2	16.14	17.85	30	93.9	5.8	7	2.0	26.89	26.31
15	167	6.9	18	4.1	0.136	32.8	0.033	1.2	16.14	19.1	31	170.0	8.1	10	1.5	20.17	17.86
16	158	6.2	18	4.6	0.162	38.9	0.041	1.5	20.17	20.83	32	—	—	10	1.5	20.17	14.2
											33	166.2	8.0	12	0.8	10.76	13.88
											34	150.7	7.8	12	0.8	10.76	12.5
											35	—	—	12	—	—	18.4
											36	—	—	16	—	—	18.45
Average.	151.3	6.95			0.151	36.3	0.034		18.71	17.05						18.6	17.02

Proportion of liver weight to body weight in the rat is 1 to 21.7. For this average only the data of normal bile collection rats were used.

secreted was great. The stasis bile was closely similar. No change from yellow to green took place such as occurs in the dog and cat, and occasionally in mouse bile after long obstruction. Though the dilatation of the ducts became greater each day, the pigment strength of their contents was never more than that of normal bile, and as a matter of fact as time passed the concentration of pigment approximated the lowest normal figures, probably as a result of dilution with fluid elaborated by the duct wall, as in the case of dogs and cats.⁶ In contrast to these results, stasis bile of the mouse always underwent a marked concentration, as will be recalled.

To find out whether there might be an early concentration of bile in the duct, followed by a later dilution as indicated, the sequence of changes in the bile after obstruction was carefully followed by means of animals killed on successive days. After the 5th day of obstruction, sufficient bile could be collected in the duct for a quantitative reading with acid alcohol, but in all cases the bichromate scale was used as with the animals killed earlier. The results in this sequence of animals are distributed amidst the earlier ones in Table V, with which they agree completely. The final average of 17.02 mg. of bilirubin per 100 cc. of bile in obstructed instances, as determined by the alcohol reagent, is remarkably similar to the figure obtained for normal bile—17.05 mg. The bichromate reading of 18.6 mg. per 100 cc. of bile also compares with the result found in non-obstructed cases of 18.7 per 100 cc. It is thus seen that rat bile undergoes no concentration after leaving the liver, even during long stasis. The secretion is about eight times as strong in pigment as mouse bile obtained from the ducts.

From these findings it is clear that there resides in the rat ducts no ability to concentrate bile such as resides in the gall bladder of the mouse. The dilatation of the rat ducts was, relatively speaking, enormous, and progressed with such regularity that one could almost state from it the number of days of obstruction. In several instances the common duct reached a diameter of 15 mm. in its broadest portion and it contained in one case more than 2.8 cc. of bile which, though still of a clear pale amber and a normal pigment content, contained more mucus than normal bile. In some animals the intrahepatic dilatation of the ducts was so great that the parenchyma had become

merely a sort of covering upon them. The liver tissue showed marked cirrhosis and was clay-yellow. Histologically the condition seen was similar to that found in dogs after long obstruction—a perilobular cirrhosis.

Influence of Diet.

The amount of bile secreted by the rat (36.3 cc. per kilo of body weight per day) and by the mouse (48.3 cc.) is not nearly as great as is put out by the rabbit and guinea pig, but is more than the output of the dog and cat. Thus, according to Quincke and Hoppe-Seyler⁸ the rabbit secretes 136.8 gm. of bile per kilo of body weight a day and the guinea pig 175.8 gm., while the dog yields but 20.0 gm. and the cat 14.5 gm. per kilo of body weight. The large bile output of the Herbivora has usually been ascribed to their diet; and our animals were on a vegetable ration. To determine the importance of this factor, several rats were placed on a meat diet for a period of 17 days, and bag collections were then made as in normal grain-fed animals. But the bile obtained was not unusual either in amount or character.

SUMMARY.

In a previous paper the point has been brought out that the influence of the gall bladder upon the bile differs entirely from that of the ducts, the one organ acting to concentrate the secretion markedly and the other to dilute it slightly. The question arises, in species lacking a gall bladder, whether the concentrating function of this organ will be found lodged in the ducts. To test the point, observations have been made upon the mouse and rat, since these animals though so nearly related have, the mouse, a gall bladder and the rat, none.

The normal bile was first studied. Both animals were found to secrete larger quantities than do cats and dogs, but less than the guinea pig and rabbit. Methods were worked out for the quantitation of the pigment which was used as the index to changes in concentration.

⁸ Quincke, H., and Hoppe-Seyler, G., in Nothnagel, H., *Specielle Pathologie und Therapie*, Vienna, 1899, xviii, 59.

Bladder bile of the mouse was regularly found to be more concentrated than that collected from the common duct of the same animal. The bile collecting during stasis regularly showed a great increase in pigment content, such as in other species is brought about by the action of the gall bladder. In the rat, on the other hand, stasis bile never became more concentrated in pigment than the normal.

The gall bladder, then, is not only absent from the rat in form, but in one at least of its important functions. That its other obvious function—that of a reservoir—cannot be assumed in the rat by the ducts would seem to be indicated, not only by the small size of these channels, but by the recent observation of Mann² that the tonus of the sphincter of Oddi is almost negligible in the rat, in contradistinction to animals which possess a gall bladder.

It is an interesting fact that the bile of the rat, which as has been said, undergoes no condensation of bulk after leaving the liver, contains on the average eight times as much pigment as does the liver bile of the mouse which is submitted to concentration. Whether it is correspondingly strong in substances useful for digestion, and therefore *ab initio* requires no concentration, is a matter upon which little can be said at present. However, in this connection the fact that the bulk of bile secreted per gram of liver weight is identical in both animals may be significant. Although this output is the same, the mouse liver when compared with the body weight (1 to 14.6) is relatively larger than that of the rat (1 to 21.7), so that the mouse secretes somewhat more bile per 100 gm. of body weight. This bile as it comes from the liver is but one-eighth as strong at least in pigment as rat bile, but the concentrating activity of the gall bladder is so great that the products yielded to the intestine may become not dissimilar.

SOURCES OF THE ANTIBODIES DEVELOPING AFTER REPEATED TRANSFUSION.

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The recent wide utilization of transfusion as a therapeutic measure has brought to light many facts of theoretical as well as practical significance. Perhaps most interesting from both points of view is the gradual decrease in beneficial effect, and the appearance sometimes of positive injury from the frequently repeated injection of alien blood. Were it not for this complication one might reasonably expect to maintain cases of pernicious anemia in good blood condition for an indefinite period of time. Needless to say, the sources of the failure have been the subject of much discussion and of some research. One fact of great weight has been clearly shown. Blood derived from a donor originally compatible, as proven both by *in vitro* tests and by the clinical result, may not only cease to be useful when too frequently injected into the same individual but may give rise to serious reactions. A change has occurred, not in the donor but in the recipient, such that the alien blood is no longer tolerated in circulation.

Boycott and Douglas¹ have noted that blood is destroyed more rapidly after repeated transfusions in normal animals than it is at first, as attested by an increase in the rate at which plethora disappears. One of us, with Oliver,² has utilized the phenomenon to induce in rabbits a hemosiderosis closely resembling that of hemochromatosis in man. No doubt a part of the pigmentation observed at autopsy in pernicious anemia patients who have been repeatedly transfused is due to a like destruction of alien blood. But how is this blood destroyed? By circulating antibodies or within special organs?

¹ Boycott, A. E., and Douglas, C. G., *J. Path. and Bact.*, 1909, xiii, 414.

² Rous, P., and Oliver, J., *J. Exp. Med.*, 1918, xxviii, 629.

Recent observations indicate that circulating antibodies are to a considerable degree involved.³ It has been found that repeated transfusions of compatible blood in rabbits are followed often by the appearance in the recipients' plasma of hemagglutinins so strong that the red cells come together into a firm mass practically as soon as the blood has been shed, while, furthermore, a fulminant destruction of corpuscles may take place *in vivo* with result in anemia. Robertson⁴ has presented evidence that the elements destroyed are the alien cells which, little by little, under the circumstances of plethora and diminished bone marrow activity consequent thereon have taken the place of cells proper to the host.

The hemagglutinins just mentioned are in the immediate sense autoantibodies. They clump practically all of the circulating erythrocytes, are especially effective at low temperatures, and persist in high titer for months after the transfusions have been discontinued and after recovery from the severe intercurrent anemia which may develop soon after their appearance. Is it possible that an organism engaged in eliminating unusually large amounts of blood will elaborate antibodies directed against its own cells? This is a point of major interest in any study of the source of the hemagglutinins, and one not without a practical bearing. In human beings true autohemagglutinins have repeatedly been observed⁵ in association with anemia of obscure origin; while autohemolysins are known to bear an important relation to paroxysmal hemoglobinuria.

Transfusion Method.

Three to six compatible donors were selected for each recipient by the examination of mixtures of the citrated bloods.⁶ The recipients received, 6 days in every 7, 10 cc. of blood taken by cardiac aspiration into 1 to 2 cc. of 0.9 per cent salt solution containing 1 per cent of sodium citrate. The donors were employed in rotation. The small amount of citrate mentioned was sufficient to prevent clotting during the short period required to introduce the blood into the recipient's ear vein. About half of the transfused animals failed to develop autoagglutinins at any time, even when the injections were continued for many weeks. In the

³ Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1918, xxvii, 509.

⁴ Robertson, O. H., *J. Exp. Med.*, 1917, xxvi, 221.

⁵ Clough, M. C., and Richter, I. M., *Bull. Johns Hopkins Hosp.*, 1918, xxix, 86.

⁶ Rous, P., and Turner, J. R., *J. Am. Med. Assn.*, 1915, lxiv, 1980.

other individuals they appeared early, as a rule after only five to ten transfusions; and when three or four more had been given, that is to say, after some 10 days to 3 weeks in all, they were well marked. The shed blood of the rabbits, when examined at room temperature, now showed prior to clotting a massive clumping of the red cells due, as our previous work has shown, to the presence in the plasma of true hemagglutinins.³

The Mixed Content of the Blood.

The transfused rabbits became, as it were, mixing vessels for several alien corpuscles and plasmas, perhaps indeed concentrators of immune principles through the elimination of the fluid wherewith the latter were introduced. The clumping of the cells of the shed blood might conceivably have been the result of:

1. The injected sodium citrate—a possibility ruled out by negative findings in control animals given citrate alone.

2. Antibodies introduced with the donor's plasma and (*a*) active against the recipient's cells. These might either have developed during the period of experiment, or have been so weak as to escape detection *in vitro*. No tests had been made to rule out (*b*) interagglutination among the donor bloods.

3. Antibodies elicited in the recipients and directed against (*a*) the strange corpuscles and (*b*) against the animal's own corpuscles.

4. Changes in the circulating cells resulting in a greater agglutinability.

5. Antibodies within the injected corpuscles, liberated by their destruction.

More remote possibilities could be invoked, as for example, a non-specific agglutination brought about through physical changes in the plasmas pooled *in vivo*, but, as will be shown, those above listed suffice to account for the findings.

Tests with Preserved Cells.

In an initial series of experiments for the analysis of the conditions, blood specimens were taken aseptically from the individuals of several donor-recipient groups prior to any transfusions, and set aside in a citrate-glucose mixture in which rabbit corpuscles remain viable

for several weeks.⁷ Later when the transfusions had elicited the clumping phenomenon, these preserved corpuscles were several times washed, made to a 5 per cent suspension with salt solution, and used in agglutination tests paralleling others carried out at the same time with freshly taken cells of the same animals.

For the purpose, equal parts of cell suspension and serum were mixed, allowed to stand for 15 to 30 minutes at room temperature, and examined microscopically. Room temperature is far more favorable to hemagglutination than blood heat. Clumping of the cells is ordinarily completed in about 10 minutes.

The tests yielded clear-cut results. Prior to transfusion no agglutination reaction was ever observed between the bloods of the prospective donors and recipients, when examined either according to the method just described or by that with whole citrated bloods previously referred to. Weak interagglutinations were sometimes noted between the bloods of certain donor groups but they were entirely absent from others. The fresh and preserved cells of the same individual were regularly found to behave almost identically on test *in vitro*. No evidence was obtained for possibility No. 4 of those above listed, namely an increase in agglutinability of cells circulating in an alien organism; but several other causes for the clumping were readily demonstrated. Most important, and most frequent, were isoagglutinins, often of high titer, developing in the recipient and effective against the cells of one or more donors (possibility 3, *a*). Such antibodies commonly appeared, and were readily demonstrable in serum separated from the clot at room temperature. Their association with the most marked clumping noted makes it highly probable that the sudden anemia supervening on plethora which occurs only in association with such marked clumping is due to isoantibodies.

Agglutinins interactive between the donor bloods (possibility 2, *b*) sometimes developed, but they were never strong. Occasionally agglutinins for the recipients' cells appeared in the donors' plasma during the period of transfusion (possibility 2, *a*). These were always so weak as to be negligible *in vivo*.

There remained a series of instances in which, with all such antibodies ruled out, there were yet hemagglutinins in the recipient that

⁷ Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

led to a marked clumping not only of the mixture of cells present after transfusion but of the recipient's cells taken prior to transfusion and kept *in vitro*. Such antibodies, like true autoagglutinins,⁸ were readily bound to, and completely freed from, the cells by cooling and warming respectively. Tests on their origin were now begun; and to rule out wholly the possibility that undetected antibodies had been injected with the donors' bloods, the transfusions were carried out with washed cells.

Transfusions with Washed Cells.

Five donor-recipient groups were chosen in the usual way and the donors bled in rotation. 10 cc. of whole blood was taken each time into about 20 cc. of a sterile isotonic solution of sodium citrate (3.8 per cent) in water and allowed to stand in the ice box for 2 days, during which period practically complete sedimentation occurred. The supernatant fluid was now pipetted off and the cells were suspended in about 20 cc. of Ringer's solution, to which had been added 0.125 per cent of gelatin and 0.2 per cent of sodium citrate. The gelatin prevented mechanical injury of the cells during the manipulations, and the citrate did away with the slight tendency to clotting. After a further 2 days of sedimentation in the cold the corpuscles were again suspended, but this time in gelatin-Ringer's solution lacking citrate, and immediately thrown down with the centrifuge; made to a total bulk of 10 cc. with an ordinary Ringer's mixture containing only 0.76 per cent NaCl; and injected. During all the handling no hemolysis occurred except occasionally to a negligible degree in the case of a donor with unusually frail cells.

Prior to the first transfusion, blood specimens from the prospective recipients were taken for preservation as already described.

In three of five rabbits repeatedly injected with the washed cells a well defined clumping in the shed blood was soon noted. In the two better marked cases the serum of the recipient, separated from the cells at 37°C., caused at room temperature outspoken agglutination of the preserved cells of the same individual. The principle which caused the red cells to come together remained fixed upon them in the cold, and the cell masses could be repeatedly washed in salt solution without its liberation. But when the fluid was warmed to body heat the agglutinins passed into it and the cell mass separated into its components. By such means the antibody proved readily obtainable in salt solution, as is the case with autoagglutinins.⁸

⁸ Landsteiner, K., *Münch. med. Woch.*, 1903, 1, 1812.

In considering these results one asks first whether they might not have been duplicated with normal blood. For it will be recalled that Landsteiner⁸ was able to demonstrate autoagglutinins in the normal blood of several animal species, among them the rabbit. But, the blood of our recipients showed no clumping prior to the transfusions. Furthermore, Landsteiner's report shows that his agglutinins were vastly weaker than ours, being demonstrable at room temperature only when a large amount of serum was allowed to act on a few corpuscles. Nevertheless, we made attempts to demonstrate autoagglutinins in five normal rabbits by the same measures that had been employed with the transfused ones, but obtained consistently negative results.

Autofixation of Isoantibodies.

A remote possibility which suggested itself to account for the autoagglutination just described was the fixation on the donor cells and persistence throughout the washing process of isoantibodies present in the donor plasma and effective against the blood of the recipient. So unlikely did this seem that we had not considered it at the time of the above mentioned transfusions, but, secure in the realization that no foreign serum would reach the recipients, had omitted compatibility tests of them and the donors. Now an experiment was undertaken to determine whether normal isoagglutinins become fixed on the possessor's own cells when these latter are allowed to sediment out of citrated plasma in the cold. Bloods suitable for the work were obtained with some difficulty owing to the fact that isoantibodies in normal rabbits are weak and rather infrequent.⁹

Experiment 1.—By the examination of mixtures of whole citrated bloods five rabbits possessing isoagglutinins were selected. In mixtures of three parts of serum separated from the clot at 37°C. and one part of 5 per cent suspension in salt solution of the susceptible cells, definite clumping occurred in all cases at room temperature. Now portions of blood from the five animals were treated to the same slow, repeated washing by sedimentation in the ice box as in the case of the donors in the transfusion experiments with washed cells already described;

⁹ Ottenberg, R., Kaliski, D. J., and Friedman, S. S., *J. Med. Research*, 1913-14, xxviii, 141.

and after the final washings, carried out with the help of the centrifuge, each cell sediment was tested as follows for serum agglutinins which might have become fixed on the corpuscles:

All possible supernatant fluid was drawn off; Locke's solution to about one-third the quantity of the original plasma was poured on; the cells were suspended for 10 minutes at 38–40°C.; and then they were thrown down by rapid, brief centrifugation. The salt solution was then immediately pipetted away and mixed in various proportions with 5 per cent suspensions of the cells that had originally been agglutinated by the plasma. As a control similar mixtures were made of cell suspensions and serum from the five animals, all kept from the time the original citrated specimens were drawn. The control serum had been removed from the clot after a few hours at incubator temperature and preserved in the ice box until needed.

In every case the salt solution clumped the cells acted upon by the control serum, while comparative tests in graded dilution showed that practically the entire original isoagglutinin content of the serum had passed into it.

The point disclosed by this experiment was most unexpected and of considerable theoretical interest. It is evident that normal isoagglutinins of the rabbit may become fixed upon the animal's own cells in the cold, to be given up again into salt solution at 37°C. Possibly in our transfusions with cells washed by sedimentation in the cold some isoagglutinins were thus carried over into the recipient. But this does not warrant the conclusion that an introduction of such isoagglutinins is the explanation of the clumping previously interpreted as autoagglutination. In the transfusions with whole citrated blood from compatible donors which resulted in what seemed to be autoagglutination the total amount of plasma injected into the recipient prior to the development of the clumping phenomenon was often considerably less and never very much more than the animal itself had to start with. Now granting that all of the alien plasma save the antibodies was removed from circulation, this would mean that the latter were left in about their original concentration in the donors, and at this concentration they had not been demonstrable *in vitro* when the donors were chosen. They cannot, then, have been responsible for the autoagglutination now seen in the recipient's shed blood and in suspensions of his preserved cells mixed with fresh serum. Nevertheless, transfusion experiments with cells washed in the warm were planned, but before they had been undertaken a new fact came to light. The corpuscles of normal rabbits

having no demonstrable isoagglutinins in the plasma were themselves found to contain such antibodies in considerable amount.

Intracellular Agglutinins.

Klein¹⁰ in 1902 claimed that extracts of red cells in salt solution or water showed agglutinative properties. His findings have met no general recognition, perhaps in part because of their conflict with the accepted rule that circulating antibodies are confined to the plasma, but also doubtless because of the irregularity of his results and the fact that the method used to obtain them is open to criticism. To obtain the agglutinins he ground the corpuscles with quartz sand; and salt solution ground with sand becomes endowed with the ability to cause a non-specific agglutination as we have repeatedly found. In the following experiment the cells were broken up by another means.

Experiment 2.—Three rabbits with plasmas that entirely failed to clump the cells of certain other rabbits in 9 to 1 and 1 to 9 mixtures of the whole bloods were bled 5 cc. each into 10 cc. of isotonic sodium citrate solution at 37°C. The corpuscles were at once packed with the centrifuge in a room maintained at body heat, and the supernatant fluid pipetted away as completely as possible, while still warm. The temperature of the room tended to go slightly above 38°C. because of the heating up of the centrifuge.

Now portions of each specimen of sedimented red cells were spread in thin layer on watch-glasses and dried in a current of air, a process requiring about 20 minutes. To the dried material of 0.7 to 1.0 cc. of cells, 1.0 to 1.7 cc. of doubly distilled water was added; and after the resulting thin paste had stood for a few minutes, it was centrifuged; the clear, deep red, supernatant fluid pipetted off; and to ten parts of this last one part of 9.0 per cent sodium chloride solution was added to restore approximate isotonicity. The fluid remained clear. It was now used in agglutination tests with corpuscles of the sort that had remained unagglutinated by the plasmas of the animals from which the cells for drying had come. Nine parts of the extract were used to one of a 50 per cent suspension of corpuscles in salt solution. In every case there was an almost immediate clumping of the cells into coarse flocculi. Under the microscope the clumping was found to be a characteristic agglutination, with a candy-like stringing out of the adherent corpuscles when pressure was made on the cover-glass, and a resumption of the original form when pressure was relieved. There was no crenation of the cells. The flocculi failed to break up when salt solution was added in quantity at room temperature, but fell apart immediately into the component cells when warmed to 37°C. in salt solution or the original cell extract; and in the latter at once reformed on cooling to or below 34.5°C.

¹⁰ Klein, A., *Wien. klin. Woch.*, 1902, xv, 413.

A closer study of the intracellular agglutinins of rabbits such as were here found should have considerable interest. We have examined the corpuscles of eight human bloods for similar principles, according to the method described, but the findings were negative.

DISCUSSION.

With the demonstration that rabbit red cells contain a principle capable of agglutinating characteristically the corpuscles of other rabbits, our study of the autoagglutination induced by transfusion came to an end. In brief, we had found that the remarkable clumping of the cells in the shed blood of repeatedly transfused rabbits is due in most instances to the action of isoagglutinins developing in the recipient and effective upon the alien elements circulating amongst its own cells. Interagglutination of the donor cells by the plasmas introduced with them may sometimes play a subsidiary part in the phenomenon, as may possibly agglutination of the recipient's corpuscles by weak antibodies appearing in the donor's plasma during the course of the experiment. There remain instances in which, with all such causes of clumping ruled out, the recipient's corpuscles, taken prior to transfusion and preserved *in vitro*, are agglutinated by the animal's own serum obtained after several injections of alien blood. That these are genuine instances of induced autoagglutination remains uncertain because the possibility was not excluded that intracellular agglutinins derived from the donors underwent liberation in the recipient. The best evidence for a true autoagglutination lies in the not infrequent long persistence of the antibodies in the recipient's blood. In one such instance the clumping phenomenon remained well marked for at least 133 days after the last transfusion. During this period the plethora consequent on the introduction of the blood had been quickly succeeded by a profound anemia during which the hemoglobin fell to 27 per cent (Palmer), and this in turn was followed by a rapid recovery to normal and many weeks of good health.³ Here the destruction and replacement of the circulating elements gave assurance that whatever the original source of the agglutinin, it was directed against the rabbit's own cells. An antibody of foreign origin would scarcely have remained so long in the blood stream. Tests with serum taken from other recipients when

the clumping phenomenon was at its height, and preserved in the ice box, reinforce such findings. Thus, for example, it was observed that cells obtained from a recipient 86 days after the last transfusion, with an intervening plethora-anemia-recovery sequence, were agglutinated by serum from the animal preserved all this while. There are several facts which speak for the view that true autoagglutinins were induced in the transfused rabbits as a by-product to isoagglutinins. Our experiment in which normal isoagglutinins became fixed in the cold on their possessor's own cells shows how close is the antigenic relationship between the erythrocytes of different rabbits. And Ottenberg and Thalheimer¹¹ have made the significant observation with regard to the normal autoagglutinins of cats that: "Those strongly agglutinative sera which affect the red cells of a large number of other animals are usually those which are auto-agglutinative." The fact that the normal isoantibodies of cats are weak at most, while isohemolysins are entirely absent from the plasma would seem to indicate that, as in rabbits, the antigenic differentiation of the red cells is but slight. Numerous recent authors have noted that immunization with a single antigen may cause the development of antibodies of rather wide application. One need only suppose for the present case that this widening of application is such that the antibodies find a mark in the animal's own cells, as was Ehrlich's original conception.

The clumping observed in the shed blood of transfused rabbits never fails to bring together practically all of the cells. Yet our analysis of the content of the sera has shown in a number of instances that agglutinins were present only for certain cells in the circulating mixture. The apparent contradiction here seen is explained by the distribution of the susceptible cells within rouleaux. These latter, which fail to break up save when agglutination is strong, are brought together with the cells. If they have been broken up by washing with warm salt solution, and the cells dispersed, the agglutination becomes selective. Whenever the clumping of the shed blood is strong enough to disorganize the rouleaux and mass all the cells confusedly, agglutinins for all are regularly found to be present on serum analysis.

¹¹ Ottenberg, R., and Thalheimer, W., *J. Med. Research*, 1915-16, xxxiii, 213.

The fact that repeated subcutaneous or intraperitoneal injections with a compatible blood will cause the development of antibodies effective against such blood has been generally accepted since Ehrlich's study of induced isolysins in goats; but it has failed of recognition in its important practical bearing on the outcome of repeated transfusions in man. Our experience with rabbits repeatedly injected from compatible donors, especially such animals as showed a sudden and great blood destruction, serves to illustrate in little what must not infrequently occur in human beings. It follows that the most careful blood tests are called for with patients repeatedly retransfused at short intervals. In special, a thick spread of the patient's blood should be examined for autoagglutination which is often, as we have shown, an evidence that newly developed isoagglutinins are in circulation. Throughout the tests the temperature factor should be carefully controlled. Serum separated from the clot at 37°C. will often yield antibodies not demonstrable in that taken in the cold, and conversely agglutination mixtures examined at room temperature are far more likely to yield positive findings than those kept at blood heat.

Whether massive transfusions are preferable in pernicious anemia to repeated small ones remains uncertain. Sudden large increments of blood tend to lessen the reparative activity of the bone marrow.⁴ But, on the other hand, they may also act to prevent fulminant destruction of the introduced blood by the recipient's serum antibodies. In the work already mentioned on experimental hemosiderosis in rabbits² some very large transfusions were given to animals that had developed strong serum antibodies, in the hope that abundant and continued blood destruction would ensue. This was never the case. The amount of antigen introduced so greatly exceeded that of the circulating antibodies that these latter failed to be destructive and plethora was maintained. It is a curious fact that in animals transfused 6 days out of every 7 during a period of 6 months the agglutinins gradually and completely disappeared, although following the first few injections they were often strong. A similar disappearance of precipitins upon long immunization has been recorded by Tschistowitsch and Nuttall.¹² Yet the transfused blood is somehow destroyed with great rapidity in animals frequently injected.

¹² Nuttall, G. H. F., *Blood immunity and blood relationship*, Cambridge, 1904, 127.

SUMMARY.

The massive agglutination observable in the shed blood of transfused rabbits, and associated not infrequently with sudden marked blood destruction, has a practical significance in connection with the untoward results of repeated transfusion from donors originally compatible; and it has special theoretical interest because the clumping of the cells is apparently an autoagglutination. To determine the actual source of the antibodies has been the object of the present work.

The agglutination in its most marked form has been traced to isoantibodies elicited by the presence in the body of corpuscles originally found compatible; and the frequently associated, rapid blood destruction is doubtless of similar origin. Occasionally antibodies develop in the donor bloods during the period of transfusion, but they are so weak as to be negligible. There remain instances of what would seem to be true autoagglutination due to serum bodies induced by the transfusions as a by-product, so to speak, in the manufacture of isoagglutinins. The antigenic relationship between the red cells of different rabbits is so close that normal isoagglutinins became fixed in the cold upon their elaborator's own corpuscles.

Agglutinins exist within the red cells of rabbits—as has been claimed by Klein. They are readily demonstrable in watery extracts of the dried corpuscles. Whether similar agglutinins ever exist within human cells remains to be determined. We have not found them in the normal corpuscles.

STUDIES ON THE PNEUMONIC EXUDATE.

V. THE RELATION OF PNEUMONIC LUNG PROTEASE ACTIVITY TO HYDROGEN ION CONCENTRATION, AND A CONSIDERATION OF THE ORIGIN OF THE ENZYME.

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(Received for publication, August 26, 1921.)

That leucocytes contain an enzyme or enzymes capable of splitting native proteins to simpler nitrogenous compounds is an accepted fact at the present time. As early as 1877 Filehne¹ succeeded in obtaining from the sputum of a case of lung gangrene by extraction with glycerol an enzyme capable of dissolving fibrin and coagulated egg white in slightly alkaline media. A few years later Stolnikow² and Escherich³ obtained similar results with extracts of sputa from cases of bronchitis, pneumonia, and phthisis. At that time the origin of the enzyme was disputed.

Müller⁴ conducted a series of experiments relative to the action of empyema and abscess pus on fibrin and coagulated egg white, and found that the substances were readily dissolved. The sputa from cases of lobar pneumonia at the time of or just following crisis had a similar effect; whereas, before the crisis no enzyme action could be demonstrated. He also noted that pieces of pneumonic lung immersed in toluene water and placed in the incubator for several days became considerably lighter. He was of the opinion that the enzyme came from the destroyed white blood corpuscles. Simon,⁵ following out Müller's views, studied chemically the autolysis of toluene water extracts of lungs from cases of lobar pneumonia. The extracts of five out of the six cases studied gave a primary acid reaction, and were subsequently neutralized or made slightly alkaline with calcium or sodium carbonate. After incubation for 5 to 8 days the average decrease in coagulable nitrogen, or increase in non-coagulable nitrogen, was 30 per cent; and an average of 30 per cent of the increase in non-coagulable nitrogen was accounted for by an

¹ Filehne, W., *Sitzungsber. physik.-med. Soc. Erlangen*, 1877, ix, 169, cited by Stolnikow.²

² Stolnikow, J., *St. Petersb. med. Woch.*, 1878, iii, 160.

³ Escherich, T., *Deutsch. Arch. klin. Med.*, 1885, xxxvii, 196.

⁴ Müller, F., *Verhandl. Cong. inn. Med.*, 1902, xx, 192.

⁵ Simon, O., *Deutsch. Arch. klin. Med.*, 1901, lxx, 604.

actual increase in albumoses. The lung of the sixth case was in the stage of red hepatization; the extract was alkaline in reaction and underwent no autolysis. Control experiments on normal lung extracts showed no autolysis. About the same time Achalme⁶ working with pus from various localities demonstrated the presence of a proteolytic enzyme capable of digesting fibrin and casein. He, also, concluded that this enzyme was derived from the leucocytes.

More recently Opie⁷ has carefully studied the proteolytic enzymes of sterile inflammatory exudates produced by intrapleural injections of an aleuronat suspension in rabbits and dogs. He concluded that there were two distinct proteolytic enzymes present in the exudates—a leucoprotease derived from the polymorphonuclear leucocytes and acting best in slightly alkaline media (0.2 per cent sodium bicarbonate), and a lymphoprotease derived from the mononuclear cells and acting best in slightly acid media (0.2 per cent acetic acid). The action of both enzymes was inhibited by the antiferment of normal beef serum. Jobling and Strouse⁸ repeated some of Opie's work and came to essentially the same conclusions except that they believed there was also an erepsin-like enzyme present, active in both acid (0.2 per cent acetic acid) and alkaline (0.2 per cent sodium bicarbonate) media. Dernby⁹ demonstrated in leucocytic suspensions enzymes capable of splitting peptone (optimum pH 7.6 to 8.0).

In a series of investigations on pneumonic exudates undertaken in this laboratory Lord¹⁰ with cellular suspensions from pneumonic lungs ranging from pH 3.1 to 7.3 obtained visible erosion on Löffler's blood serum only at the higher end of the scale (pH 6.7 to 7.3). Peptone solutions were appreciably split by these cellular suspensions, and most complete splitting occurred at pH 5.2 or 6.3. He formulated the hypothesis that during the course of the disease in the pneumonic lung there is a gradual decrease in pH or increase in acidity. Primarily and at that time when the reaction of the exudate is about that of the circulating blood (pH 7.3 to 7.5) there is a digestion of the more highly organized proteins (fibrin, serum albumin, etc.) following the liberation of enzyme through cellular disintegration. With subsequent increase in acidity (beyond pH 6.7) the activity of this protease diminishes and conditions reach an optimum for the activity of the peptone-splitting enzyme. This peptonase or ereptase carries the splitting of the digestion products to amino-acids, absorbable as such; and resolution takes place.

It seemed advisable to determine quantitatively the digestive action of the exudate on one of the higher proteins and the relation of hydrogen ion concentration to the activity of the enzyme. As a

⁶ Achalme, *Compt. rend. Soc. biol.*, 1899, li, 568.

⁷ Opie, E. L., *J. Exp. Med.*, 1906, viii, 410.

⁸ Jobling, J. W., and Strouse, S., *J. Exp. Med.*, 1912, xvi, 269.

⁹ Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.

¹⁰ Lord, F. T., *J. Exp. Med.*, 1919, xxx, 379.

substrate fibrin was chosen, since it is one of the higher proteins which must be broken down in the pneumonic lung during the process of resolution. Since a cellular suspension of the consolidated lung, rather than of the exudate alone, was used, it was necessary to rule out the action of enzymes contained in normal lung tissue.

EXPERIMENTAL.

1. *Preparation of Substrate*.—A mass of fresh horse fibrin about the size of a baseball was picked and washed practically free from adhering blood clot, and then passed through a meat chopper with a nut butter cutter attachment. The mash was centrifuged and washed repeatedly with water until the supernatant fluid was clear and colorless. The water was filtered off and the fibrin dried at 100°C. over night on a steam water bath. After grinding, the yield was 2.7 gm.

2. *Preparation of Enzymes*.—Portions of gray-red hepatization and normal lung from a case of Type I lobar pneumonia had each been previously ground in a meat chopper with a nut butter cutter attachment and kept in the ice box several weeks following the addition of chloroform and toluene. The consolidated lung mash was washed through two thicknesses of sterile gauze with 0.85 per cent sterile salt solution and the washings were centrifuged and washed four or five times with salt solution. The final centrifuging (No. 7 stop for 7 minutes) yielded 5 cc. of "cells," which were made up to 15 cc. with sterile salt solution. The normal lung mash was ground with sterile sand in a sterile mortar; and, after separating the sand from the suspension by centrifuging at low speed, treated as above. The yield was only 0.6 cc. of "cells;" these were made to 15 cc. with salt solution.

3. *Method*.—To dry 60 cc. sterile flasks were added phosphate mixtures, fibrin, 10 cc. of sterile water (Nos. 6 to 15 inclusive, 15 cc.), and 1.0 cc. of cellular suspensions (Nos. 6 to 15 inclusive, 1.5 cc.) (Table I). To those flasks intended to have pH values of 4.0 and 5.0 were added 4 drops of a 2 per cent aqueous solution of sodium alizarin sulfonate, and to the remainder 2 drops of a 0.01 per cent solution of phenol red. The flasks were adjusted approximately to the desired pH by the addition of normal and 0.1 normal hydrochloric acid and sodium hydroxide and comparison with a set of standard pH solutions prepared according to Clark and Lubs.¹¹ Water was added to make the total volume 20 cc. (Nos. 6 to 15 inclusive, 30 cc.). After thorough agitation 10 cc. samples were removed from Flasks 6 to 15 inclusive. 1 cc. of toluene was added to each flask and all were tightly corked and placed in the incubator at 37.5°C. for 5 days.

Cultures taken after 12 hours incubation and at the end of the experiment were sterile. During incubation there was no appreciable change in reaction.

The coagulable proteins were precipitated by the addition of trichloroacetic acid (3 per cent of total volume) according to Greenwald.¹² The tubes were

¹¹ Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.

¹² Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

TABLE I.

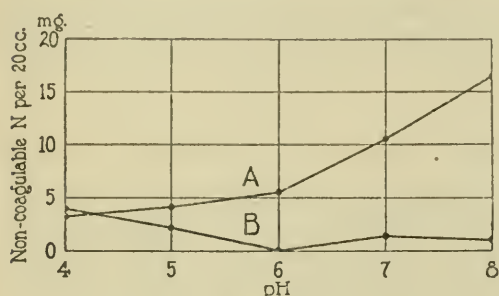
The Composition of the Various Digestion Mixtures and the Results of the Determinations.

Tube No.	Fibrin.	Pneumonic lung cellular suspension.	Normal lung cellular suspension.	0.5 M KH_2PO_4 .	0.5 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$.	Water, acid, and alkali.	pH	Non-coagulable nitrogen per 20 cc.			Amino nitrogen Per 20 cc.		$\frac{\text{Amino nitrogen}}{\text{Non-coagulable nitrogen}} \times 100.$
								Before digestion.	After digestion.	Actual digestion.	After digestion.	Actual digestion.	
	gm.	cc.	cc.	cc.	cc.	cc.		mg.	mg.	mg.	mg.	mg.	per cent
1	0.30*			2.0		18.0	4.0	—	1.9	—	0.46	—	
2	0.30			1.8	0.2	18.0	5.0	—	2.5	—	0.50	—	
3	0.30			1.6	0.4	18.0	6.0	—	4.2	—	0.52	—	
4	0.30			0.4	1.6	18.0	7.0	—	5.4	—	0.62	—	
5	0.30				2.0	18.0	8.0	—	6.3	—	0.74	—	
6		1.5		3.0		25.5	4.0	1.0	1.2	0.2	0.24	—	
7		1.5		2.7	0.3	25.5	5.0	0.6	1.0	0.4	0.28	—	
8		1.5		2.4	0.6	25.5	6.0	0.8	1.7	0.9	0.34	—	
9		1.5		0.6	2.4	25.5	7.0	0.5	2.0	1.5	0.54	—	
10		1.5			3.0	25.5	8.0	0.7	2.5	1.8	0.56	—	
11			1.5	3.0		25.5	4.0	0.1	0.3	0.2	—	—	
12			1.5	2.7	0.3	25.5	5.0	0.1	Lost.	?	—	—	
13			1.5	2.4	0.6	25.5	6.0	0.2	0.8	0.6	—	—	
14			1.5	0.6	2.4	25.5	7.0	0.2	0.3	0.1	—	—	
15			1.5		3.0	25.5	8.0	0.2	0.6	0.4	—	—	
16	0.30	1.0		2.0		17.0	4.0	—	5.8	2.7	1.02	0.32	12
17	0.30	1.0		1.8	0.2	17.0	5.0	—	7.7	4.2	1.22	0.44	11
18	0.30	1.0		1.6	0.4	17.0	6.0	—	11.3	5.4	1.58	0.72	13
19	0.30	1.0		0.4	1.6	17.0	7.0	—	17.9	10.5	2.76	1.60	15
20	0.30	1.0			2.0	17.0	8.0	—	25.2	16.4	3.26	1.96	12
21	0.30		1.0	2.0		17.0	4.0	—	6.2	4.0	—	—	
22	0.30		1.0	1.8	0.2	17.0	5.0	—	4.6	1.6†	—	—	
23	0.30		1.0	1.6	0.4	17.0	6.0	—	5.0	0.0	—	—	
24	0.30		1.0	0.4	1.6	17.0	7.0	—	7.1	1.4	—	—	
25	0.30		1.0		2.0	17.0	8.0	—	7.8	0.9	—	—	

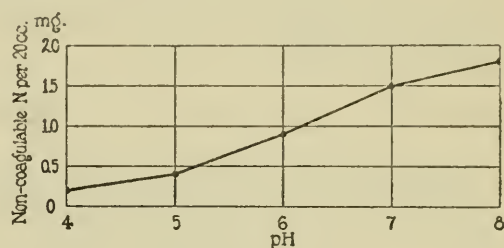
* Total nitrogen determination 0.042 gm.

† Estimating No. 12 flask after digestion as 0.5 mg.

stoppered, shaken, and allowed to stand 12 to 15 hours in the ice box before filtering. Nitrogen determinations on the filtrates were made by Folin's¹³ macro Kjeldahl method, using 5 cc. samples and 5 cc. of his digestion mixture. Duplicate determinations were made on all filtrates. The non-coagulable nitrogen representing the actual digestion of the fibrin by the cellular enzymes was obtained by subtracting the sum of the non-coagulable nitrogen of the fibrin and lung controls from that of the digestion mixture. The results are expressed as the average amount of non-coagulable nitrogen present in 20 cc. Amino nitrogen was determined by Van Slyke's¹⁴ nitrous acid method using 2 cc. samples of the filtrates following the precipitation of the coagulable nitrogen. Blank determinations made with distilled water and with distilled water saturated with toluene eliminated the presence of traces of the preservative as a source of error. Results are expressed as the amount of amino-acid nitrogen present in 20 cc.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Proteolytic activity of pneumonic (A) and normal (B) lung cellular suspensions at varying hydrogen ion concentrations.

TEXT-FIG. 2. Autolysis of pneumonic lung cellular suspensions at varying hydrogen ion concentrations.

The results clearly show (Text-fig. 1) that there is present in the pneumonic lung an enzyme, or enzymes, capable of digesting horse fibrin. Similar results were obtained with cellular suspensions from the consolidated lungs of lobar pneumonias other than those caused by Type I pneumococcus. In the range of reactions chosen as physiological possibilities the enzyme shows the greatest activity at the most alkaline end of the scale (pH 8.0). At this hydrogen ion concentration it was able to convert 46 per cent¹⁵ of the fibrin origi-

¹³ Folin, O., and Wright, L. E., *J. Biol. Chem.*, 1919, xxxviii, 461.

¹⁴ Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 121.

¹⁵ Calculated from the following figures (Table I): total nitrogen of fibrin, 42 mg.; non-coagulable nitrogen of fibrin at pH 8.0, 6.3 mg.; and actual increase in non-coagulable nitrogen in digestion mixture at pH 8.0, 16.4 mg.

nally present to a non-coagulable form after 5 days incubation. The digestion at pH 4.0 is comparatively slight; and there is a gradual increase of digestion corresponding to decrease in acidity. Appreciable autolysis of the pneumonic lung controls occurred, slight at pH 4.0 and 5.0 with a gradual increase towards the alkaline side (Text-fig. 2). Presumably the same enzyme is responsible for both digestive processes. The amino-acid nitrogen determinations show that in the pH 7.0 digestion mixture the protein splitting was most complete. This corresponds roughly to the optimum hydrogen ion concentration of the pneumonic lung peptonases or ereptases, as reported by Lord¹⁰ and confirmed in a paper to follow. The cellular suspension controls of normal lung show an appreciable digestion of the fibrin at pH 4.0, and practically no increase in non-coagulable nitrogen in the less acid media (Text-fig. 1).

DISCUSSION.

This protease can be conceived as arising from one or more of four sources: (a) the pneumococci, (b) the cells present in the normal lung, (c) the blood serum, and (d) the cells of the pneumonic exudate.

Avery and Cullen¹⁶ have been able to obtain from pneumococci intracellular enzymes capable of digesting fibrin. With fibrin freshly prepared from rabbit blood and the bile extract from the organisms contained in 200 cc. of a plain bouillon culture of *Pneumococcus* Type II, the total actual digestion at pH 7.4 amounted to an increase of 0.26 mg. of amino nitrogen. With 0.2 gm. of commercial fibrin and a similar amount of enzyme, their actual increase in amino nitrogen was only 0.11 mg. Furthermore, since their enzyme contained non-coagulable nitrogen and in addition underwent autolysis on incubation, it is conceivable that in their digestion mixtures a portion of the increase in amino nitrogen might have been due not to an enzyme digesting native protein but to one autolyzing the enzymatic non-coagulable nitrogen—a peptonase, the presence of which they clearly demonstrated in extracts of the pneumococcus. With 0.3 gm. of fibrin and 0.33 cc. of washed cellular suspension from the pneumonic lung the total increase in amino nitrogen at pH 7.4 fell somewhere between 1.60 mg. (pH 7.0) and 1.97 mg. (pH 8.0). Stained

¹⁶ Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 547.

smears of the cellular suspension showed no intact organisms. In the washing of the cellular material in preparation one would expect the enzyme liberated from the disintegrated pneumococci to have been lost. Undoubtedly the pneumococcus protease contained in 0.33 cc. of washed cellular suspension is negligible when considering digestion amounting to 1.60 to 1.97 mg. of amino-acid nitrogen.

Opie⁷ has shown that sterile pleural exudates containing an excess of mononuclear cells exhibit maximum proteolytic activity in slightly acid media (0.2 per cent acetic acid). The phagocytic mononuclear or endothelial cells are derived from the endothelial cells lining the blood and lymph vessels.¹⁷ A cellular suspension obtained from a normal lung, or to a less degree from the true lung substance of a pneumonic lung, would contain, so far as those cells which are known to contain proteolytic enzymes are concerned, chiefly endothelial cells. Consequently from Opie's work one would expect maximum digestion in a slightly acid medium. This was exactly what happened in the normal lung cellular suspension and fibrin digestion mixtures. Since nearly twice as much fibrin was digested at pH 4.0 by the 0.04 cc. of normal lung cellular suspension as by the 0.33 cc. of pneumonic cellular suspension, it can be safely assumed that the latter did not contain as much true lung substance as the former. In spite of the presence of at least an equal amount of true lung substance, the normal lung suspension as compared with that of the pneumonic lung showed but slight digestion in the slightly acid, neutral, and slightly alkaline digestion mixtures. Hence, the enzyme, or enzymes, derived from the true lung substance of a pneumonic lung play but a slight rôle in the proteolysis of fibrin by the pneumonic lung cellular suspension except in moderately acid media (pH 4.0).

Proteases or trypsin-like ferments are known to be normally present in blood serum; but they are held in abeyance by the presence of antitrypsin. Delezenne and Pozerski¹⁸ observed that the anti-tryptic power of the serum is lost following extraction with chloroform. It is conceivable that in lung cellular suspensions preserved with chloroform and toluene the serum proteases become potentially

¹⁷ Mallory, F. B., *The principles of pathologic histology*, Philadelphia and London, 1918.

¹⁸ Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, *lv*, 327, 690.

active. However, frequent washings in preparation of the enzyme previous to the experiment would certainly remove them.

The protease of pneumonic lung cellular suspensions is derived chiefly from the leucocytes of the exudate. The other possible sources play but a minor rôle. Confirming the work of many other investigators, it was found that this enzyme is most active in a slightly alkaline medium (pH 8.0).

SUMMARY.

1. Washed cellular suspensions of pneumonic lungs, previously preserved with chloroform and toluene, contain a protease or proteolytic ferment, derived chiefly from the leucocytes of the exudate.

2. This protease is able to convert horse fibrin to a non-coagulable split product. In a pH range of 4.0 to 8.0 the digestion is slight at the most acid end. With decrease in acidity there is a gradual rise in activity to pH 6.0, and then a sharp increase up to the maximum digestion at pH 8.0. Judging from the amino nitrogen determinations, the most complete splitting occurs at pH 7.0. The degree of autolysis occurring in the pneumonic lung cellular suspension controls suggests that the active enzyme is identical with that causing the digestion of the fibrin.

3. Washed cellular suspensions of normal lungs, previously preserved with chloroform and toluene, contain a proteolytic enzyme which is active in a moderately acid medium (pH 4.0) and essentially inactive in less acid, neutral, and slightly alkaline media.

The author is indebted to Dr. F. T. Lord for many helpful suggestions.

THE TRANSMISSION OF AGGLUTININS OF BACILLUS ABORTUS FROM COW TO CALF IN THE COLOSTRUM.

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In the work on infectious abortion associated with *Bacillus abortus* carried on in this Department since 1917,^{1,2} the blood serum and transudates of fetuses have been tested from time to time to determine

TABLE I.
Agglutinin Content of Blood of Dam and Aborted Fetus.

Case No.	Blood serum.		<i>B. abortus</i> isolated.* <i>Fetus</i>	Remarks.
	Dam.	Fetus.		
493	1:1,280	1:40	Yes.	1:20 to 1:80 show the same degrees of agglutination. Abdominal and thoracic transudates negative.
374	1:1,280	1:80	"	
652	1:1,280	0	"	
506	?	0	"	
270	1:1,280	0	"	
590	1:320	1:40	"	
595	1:320	1:20	"	
579	1:80	1:20	No.	
509	?	1:40	Yes.	
518	1:1,280	1:20	"	
668	1:320	1:20	"	
671	1:1,280	1:20	"	

* From autopsy records of the Department.

the relation between the concentration of agglutinins in the blood serum of mother and fetus. The results, given in Table I, indicate that even when the serum of the mother has a high agglutinin content,

¹ Smith, Theobald, *J. Exp. Med.*, 1919, xxx, 325.

² Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx 341

little or none is found in the fetal blood. The agglutinin figures given represent the agglutination limits of series of dilutions.

Tests of the blood of calves within the first few days of life presented different results. In some a relatively high titer was observed. The variation from case to case was finally explained by the absorption of agglutinins in the colostrum, as the experiments reported below amply demonstrate.

The literature on the transmission of various antibodies in the milk is fairly rich. Only a few publications bear more directly on the subject in hand. For a thorough review up to 1912 the reader is referred to Famulener's article.³ In his own investigations on this subject, Famulener immunized goats with sheep blood corpuscles. He tested the antibody content of the mother's serum and colostrum at time of parturition and the blood of the kids before and after they had ingested colostrum. He found that in immunized goats the colostrum was rich in antibodies but in the kids no antibodies were present until after taking the colostrum. He observed that the titer of the colostrum in many cases was above that of the mother's serum, and that the milk quickly dropped in antibody content. From his experiments he concluded that the hemolytic antibodies in the young are received through the colostrum and absorbed by the young from the gastrointestinal tract, but that this absorption can only take place during the first few days after birth.

Rettger and White⁴ in 1918 and Rettger⁵ in 1920 made the statement that, without exception, cows with a positive agglutination to *B. abortus* gave birth to calves which showed the same reaction. This statement is true only after the first feeding with colostrum.

Recently Reymann⁶ in studying the transfer of normal agglutinins from mother to young also worked with goats, but he brings out no new points concerning the transmission of agglutinins. Strangely enough he fails to refer to Famulener's results.

EXPERIMENTAL WORK.

The experiments to be reported differ from those of others in that they deal with the naturally acquired and stored agglutinins as the result of a definite infectious disease of cows, localized in the fetal membranes. Earlier experiments deal only with agglutinins experimentally induced.

³ Famulener, L. W., *J. Infect. Dis.*, 1912, x, 332.

⁴ Rettger, L. F., and White, G. C., *Storrs Agric. Exp. Station, Bull.* 93, 1918, 225.

⁵ Rettger, L. F., *Abstr. Bact.*, 1920, iv, 13.

⁶ Reymann, G. C., *J. Immunol.*, 1920, v, 227.

The procedure for the collection and dilution of the blood serum for the agglutination tests is the same as described in a previous paper.² The milk was handled as follows:

The samples were centrifuged and the cream layer was removed. This was necessary in order to obtain a clear whey; however, with some samples of colostrum which were very thick the centrifuging was omitted. The samples were then warmed to about 35°C. and several drops (8 to 10) of a solution of a rennet tablet in sterile distilled water were added. The tubes were well shaken and left standing in the water bath 15 to 20 minutes. By that time a firm coagulum had formed. In order to separate the whey quickly, the tubes were then put into warmer water, at about 45°C. This caused the coagulum to shrink and a clear whey to separate. This was drawn off and used for the agglutination tests. Frequent tests have shown that the rennet does not influence the agglutinins present.

The preparation and standardization of the antigen for blood and milk was as follows:

Bacillus abortus was grown on agar at 37°C. for 24 to 48 hours. The growth was washed off in 0.85 per cent salt solution. This was standardized to a given opacity by means of the Gates instrument.⁷ A depth of disappearance of 2.4 cm. was taken as the standard. Each lot of antigen was made up to this density before use.

In Table II the agglutination titers of the blood and colostrum of seven cows and their calves soon after birth are given to illustrate the conditions usually found. Of these offspring, three (Nos. 488, 619, and 484A) were tested after suckling and agglutinins found in their blood. Two were negative when tested before suckling (Nos. 484B and 625). Of two premature calves, one had agglutinins in the blood (No. 567), the other not (No. 483).

These random illustrations showed the need of determining by suitably controlled tests the relation of the ingestion of colostrum to the agglutinin titer. In Table III illustrations of such tests are given. In the first case agglutinins appeared in the blood of the calf after it had suckled a cow (not its mother) whose colostrum had a high titer. In the second, the calf was fed colostrum artificially and agglutinins appeared but of a lower titer than in the preceding calf

⁷ Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

TABLE II.

Material tested.	Date of blood test.	Dilutions.										Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
Cow 484 and twin calves A and B, born Mar. 23, 1921. A had nursed before the first bleeding; B, much weaker, had not nursed until after the first bleeding.												
	1921											
Cow's blood.....	Mar. 23	C.*	C.	+++	+	—	—	—	—	—	—	—
Colostrum.....	" 23	"	+++	+++	++	+	—	—	—	—	—	—
Blood, Calf A.....	" 23	+++	+	+	—	—	—	—	—	—	—	—
" ".....	" 24	C.	+++	+	+	—	—	—	—	—	—	—
" " B.....	" 23	—	—	—	—	—	—	—	—	—	—	—
" ".....	" 24	C.	++	+	—	—	—	—	—	—	—	—
Cow 488 and calf which had nursed before blood test. Calf born Feb. 18, 1921.												
Cow's blood.....	Feb. 18	C.	C.	C.	+++	+++	++	++	—	—	—	—
Colostrum.....	" 18	+++	+++	+++	+++	+++	C.	C.	+	+	+	—
Calf's blood.....	" 18	+++	+++	+++	+++	+++	+++	+++	++	+	—	—
" ".....	" 19	C.	+++	+	++	C.	+	+	++	++	+	—
Cow 619 and calf which had nursed before first bleeding. Calf born June 9, 1921.												
Cow's blood.....	June 9	C.	C.	C.	C.	C.	C.	C.	++	++	++	—
Colostrum.....	" 9	"	"	"	"	"	"	++	++	++	++	—
Calf's blood.....	" 9	"	+++	+++	+++	+++	+++	+++	++	++	++	—
" ".....	" 10	+++	+++	C.	C.	C.	C.	C.	++	++	++	—

Cow 625 and calf which had not nursed before first bleeding. Calf born July 3, 1921.

Cow's blood.....	July 5	C.	C.	C.	C.	++	++	++	++	—	—
Calf's ".....	" 3	—	—	—	—	—	—	—	—	—	—
" ".....	" 4	C.	C.	+++	++	++	+	±	—	—	—

Cow 483 and 8 mo. calf (premature) which had not nursed before first bleeding. Calf born Mar. 5, 1921.

Cow's blood.....	Mar. 5	C.	C.	C.	C.	++	++	++	++	++	—
Colostrum.....	" 5	++	++	++	++	++	++	++	++	++	—
Calf's blood.....	" 5	—	—	—	—	—	—	—	—	—	—
" ".....	" 6	++	+	+	—	—	—	—	—	—	—
" ".....	" 7	++	+	+	—	—	—	—	—	—	—

Cow 477 and calf which may have nursed before bleeding.

Cow's blood.....	May 28	C.	C.	C.	C.	++	++	+	—	—	—
Colostrum.....	" 28	++	++	++	++	++	++	+	—	—	—
Calf's blood.....	" 28	+	—	—	—	—	—	—	—	—	—
" ".....	" 31	++	+	+	—	—	—	—	—	—	—

Cow 567 and premature unfed calf. *B. abortus* found in the organs subsequently.

Cow's blood.....	Feb. 16	C.	C.	C.	C.	++	++	++	++	—	—
Colostrum.....	" 16	++	++	++	++	++	++	++	++	—	—
Blood of fetus.....	" 16	++	++	++	++	++	+	—	—	—	—

* The plus signs stand for degrees of clumping from almost complete (+++++) to just visible clumping (+). The minus sign means less than 1:20, and no clumping in controls. The letter C stands for complete clearing of the fluid.

TABLE III.

No. of dam.	Titer.		Age of calf when blood was tested.	Titer of blood.	Remarks.
	Blood.	Colostrum.			
Calf of Cow 627, born July 7, 1921, at 9.40 a.m. Restrained from taking colostrum until after three examinations of the blood, then allowed to suckle a high titer cow (No. 626) which had given birth to a calf the same day.					
627	1:160	1:320	1 hr., 50 min. 7 hrs., 10 " 21 " 30 "	— — —	Calf received 1 quart of milk free from agglutinins.
Calf placed with Cow 626.					
626	1:1,280	1:2,560	32 hrs. 46 " 50 min.	1:640 1:640	
Calf born of a high titer cow (No. 683), July 21, 1921, at 12.30 p.m. Restrained from taking colostrum until after two examinations of blood, then fed dam's colostrum which had been refrigerated and warmed.					
683	1:1,280	1:2,560	4 hrs., 45 min. 21 " 10 "	— —	Calf received 42 ounces of milk free from agglutinins.
			Calf received 36 ounces of colostrum.		
			28 hrs., 30 min. 45 " 10 "	1:40 1:160	Calf received 92 ounces of colostrum.
Calf born of a high titer cow (No. 634), July 12, 1921, at 11 a.m. Restrained from taking colostrum until after three blood examinations, then fed dam's colostrum which had been refrigerated and warmed.					
634	1:320	1:640	1 hr. 6 hrs. 22 " 30 min.	— — —	Calf received 1 quart of milk free from agglutinins.
			Calf received 33 ounces of colostrum.		
			29 hrs. 45 "	— —	
Calf born of a high titer cow (No. 657), Sept. 16, 1921, at 4.20 p.m. Restrained from taking colostrum or milk until after two examinations of the blood, then fed milk free from agglutinins.					
657	1:1,280	1:1,280	40 min. 16 hrs., 40 "	— —	
			Calf received milk.		
			2 days, 16 hrs., 40 min. 5 " 16 " 40 "	— —	

TABLE III—*Concluded.*

No. of dam.	Titer.		Age of calf when blood was tested.	Titer of blood.	Remarks.
	Blood.	Colostrum.			
Calf born of a low titer cow (No. 653), July 3, 1921, at 2 p.m. Restrained from taking colostrum until after one examination of the blood, then allowed to suckle a high titer cow (No. 654) which had given birth to a calf the same day. Milk was collected from Cow 653, 4 days after parturition, and blood 25 days after.					
653	1:20	1:160	1 hr., 35 min.	—	
	1:20	— (milk)			
Calf placed with Cow 654.					
654	1:320	1:1,280	18 hrs., 50 min.	1:320	
Calf born of a high titer cow (No. 655), July 23, 1921, at 5.30 a.m. Restrained from suckling until after one blood examination, then allowed to suckle dam.					
655	1:640	1:1,280	5 hrs., 5 min.	—	
		Calf placed with Cow 655.			
			11 hrs., 20 min.	1:160	
		32 " 10 "	1:640		
Calf born of a high titer cow (No. 684), July 22, 1921, at 11.30 a.m. Restrained from suckling until after two blood examinations, then allowed to suckle dam.					
684	1:640	1:1,280	2 hrs., 15 min.	—	
			6 " 10 "	—	
		Calf placed with Cow 684.			
			11 hrs., 50 min.	1:160	
		23 " 15 "	1:320		

which suckled naturally. In the third illustration, the calf although fed colostrum failed to show agglutinins in its blood. In the fourth case, the colostrum of the mother had a high titer but the calf's blood was negative and remained so for nearly 6 days while the colostrum was being withheld. Later tests were interfered with by the death of the calf. The remaining cases reenforce the data given in the others. In the seventh, the agglutinins in the blood of the calf rose quite rapidly from the 11th to the 23rd hour.

In Table IV, the results of agglutination tests on the blood serum and colostrum of the mother and the blood serum of the new-born calf before and after the first meal of colostrum are presented in more detail to add to the data already presented and to show certain slight

irregularities in the flocculation of *Bacillus abortus* in the series of tubes containing dilutions of the colostrum and of the blood serum of the calf. These irregularities are evident in the calf's blood serum and colostrum of No. 669 and in the colostrum of No. 411. Similar irregularities are shown in Table II (Nos. 488 and 619).

In three cases of Table IV, the agglutinin titers of blood serum and colostrum of the dam were high but that of the calf's serum was negative until after the first meal. The fourth case (No. 664) presents a lower titer but the transmission of agglutinins to the calf is nevertheless in evidence. The higher concentrations of agglutinins in the colostrum than in the blood serum of the mother is indicated in three out of the four cases. In two cases the titer of the calf's serum rose above that of the mother.

DISCUSSION.

The tabulated observations and experiments are sufficient to establish the fact that even when the blood and colostrum of the dam have a relatively high content of agglutinins towards *Bacillus abortus*, the blood of the new-born calf, with rare exceptions, is free from such antibodies until the animal has suckled and taken in the colostrum. The only exception found is the last case in Table II. In this premature calf the autopsy record shows that the stomachs were still free from food and that *Bacillus abortus* was present in the spleen, liver, and kidneys. The antibodies which appear in the calf's blood are absorbed from the digestive tract. The precise age of the calf after which agglutinins fail to enter the blood as such has not been determined. Experiments bearing on this aspect of the subject are under way.

When colostrum was withheld and milk of a low or negative agglutinin titer substituted, agglutinins failed to appear or accumulate in the blood of the calf. The longest period following birth during which the colostrum was withheld in the various tests was 5 days.

A study of the tables shows, what has been pointed out by others, that the concentration of the agglutinins in the colostrum may exceed that of the blood at and immediately after parturition.

The rate of absorption into the blood is shown to be fairly rapid. Agglutinins began to appear slightly over an hour after the calf

had ingested colostrum. They were nearly at the maximum concentration 5 hours after feeding.

The test upon all samples was carried out as shown in detail in Table IV. The dilutions were made in series from 1:20 to 1:1,280 or 1:2,560, each tube being one-half the dilution of the preceding tube of the series. By this method certain irregularities in the agglutinin concentration in the colostrum were occasionally encountered. In certain tubes of a series the flocculation was stronger than in a higher, preceding concentration. In such cases the irregularities may appear in the blood serum of the calf after taking colostrum, but the blood serum of the mother does not present them.

CONCLUSIONS.

The agglutinins towards *Bacillus abortus* found in the blood serum of new-born calves are obtained from the mother through the colostrum. Calves at birth, unfed, are without agglutinins. The problem of the production of agglutinins by the fetus in whose tissues *Bacillus abortus* has multiplied and which is subsequently expelled prematurely is not touched by these observations.

THE ERYTHROPOIETIC ACTION OF GERMANIUM DIOXIDE.

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Not only have the substances which by their commonness, such as the salts of sodium, potassium, and calcium, or by their striking and unexpected action on living tissues, such as radium and its derivatives, been intensively studied, but also many observations have been reported of the therapeutic trials and physiological action of the rarer elements. Cerium,¹ cesium,² erbium,³ indium,⁴ lanthanum,⁵ neodmiumy,⁵ palladium,⁶ praseodymium,¹ rhodium,⁷ rubidium,⁸ ruthenium,⁹ samarium,¹⁰ selenium,¹¹ tellurium,¹² thallium,¹³ thorium,¹⁴ titanium,¹⁵ vanadium,¹⁶ yttrium,¹⁷ and zirconium¹⁸ have all received some attention from the physiological point of view.

¹ Frouin, A., *Compt. rend. Acad.*, 1920, clxx, 1471.

² Kaiser, L., *Arch. néer. physiol.*, 1918-19, iii, 587.

³ Mines, G. R., *J. Physiol.*, 1910, xl, p. xlviii.

⁴ Gerber, G., *Compt. rend. Soc. biol.*, 1910, lxix, pt. 2, 104.

⁵ Rénon, L., *Bull. et mém. Soc. méd. hôp. Paris*, 1920-21, xlv, 602.

⁶ Ascoli, M., and Izar, G., *Biochem. Z.*, 1907, vi, 192.

⁷ Rémond, A., *Schweiz. Apoth. Z.*, 1919, lvii, 242.

⁸ Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 567.

⁹ Kalle and Co., German Patent No. 289,620, July 31, 1913.

¹⁰ Esnault and Bron, *Bull. et mém. Soc. méd. hôp. Paris*, 1920-21, xlv, 606.

¹¹ Duhamel, deB.-G., *Compt. rend. Soc. biol.*, 1919, lxxxii, 724.

¹² Shie, M. D., and Deeds, F. E., *Pub. Health Rep., U. S. P. H.*, 1920, xxxv, 939.

¹³ Pöhlmann, A., *Arch. Dermatol. u. Syph.*, 1912-13, cxiv, 633.

¹⁴ Gudzent, F., and Herschfinkel, *Strahlentherap.*, 1916, vii, 519.

¹⁵ Hanzlik, P. J., and Tarr, J., *J. Pharmacol. and Exp. Therap.*, 1920, xiv, 221.

¹⁶ von Oefele, F., *New York Med. J.*, 1913, xcvi, 78.

¹⁷ Mines, G. R., *J. Physiol.*, 1910, xl, 327.

¹⁸ Hébert, A., *Bull. Soc. chim.*, 1906, xxxv, series 3, 1299.

Germanium is absent from this list. We have been unable to find in the literature any references to therapeutic or physiological studies of this element or its compounds.

EXPERIMENTAL.

The germanium dioxide used in these experiments was supplied by Müller.¹⁹

It was found that the mature albino rat can withstand the subcutaneous injection of 180 mg. of a 0.4 per cent solution of germanium dioxide per kilo of body weight without fatal result or the appearance of any symptoms whatsoever of a toxic effect. The detailed report of these experiments is now in press.²⁰ That study was planned as a preliminary to the determination of the effect of germanium on the erythropoietic system because of the interest that has been attached to arsenic in this connection and because germanium occupies a place in the periodic system next to arsenic and in many of its reactions resembles this latter element.

For this study there were used two litters of mature albino rats. In one litter, 150 days old, there were three males and four females. In the other, which was about 175 days old, there were four males and four females. The females had been segregated before puberty and had never been pregnant. All the rats had from birth been on the same varied diet which was continued throughout the investigation. We thus had for the purposes of our experiment two groups of animals of two lots each in which constitutional variability was reduced to a minimum. The relatively low variability within the litter has been shown by Jackson²¹ and King.²² Each lot of rats was kept in a separate cage.

Two preliminary determinations, 7 days apart, were made of the erythrocyte and leucocyte counts of each rat of each lot in order to establish the normal values. One rat of each lot was reserved as control. Weekly counts of the cellular blood components were made

¹⁹ Müller, J. H., *J. Am. Chem. Soc.*, 1921, xliii, 1085.

²⁰ Hammett, F. S., Müller, J. H., and Nowrey, J. E., Jr., *J. Pharmacol. and Exp. Therap.*, 1922 (in press).

²¹ Jackson, C. M., *Am. J. Anat.*, 1913-14, xv, 1.

²² King, H. D., *Anat. Rec.*, 1915, ix, 751.

of all the rats—controls and tests. The blood on which the counts were made was obtained by cutting off a bit of the tail for each determination. The blood was allowed to flow freely and the first 1 or 2 drops were discarded. This was done under light ether anesthesia as were the injections of the germanium dioxide solution.

The test rats of the first group, both males and females, were injected with 1.2, 1.5, 1.8, and 2.1 mg. of germanium dioxide per kilo of body weight in sterile 0.4 per cent solution on the 8th, 11th, 15th, and 19th days of observation respectively. The solution was made at first slightly alkaline in order to facilitate the dissolving of the oxide. It was then brought back to neutrality by dilute hydrochloric acid. The rats of the second group were injected with ten times the amount given to the first group, on the 8th, 11th, and 15th days of observation. No injections were made on the 19th day because an increased coagulability of the blood had appeared which made difficult the taking of a proper sample. A total of 6.6 mg. per kilo of body weight was injected into the rats of the first group and of 45 mg. into the test rats of the second group.

Two more counts were made of the corpuscles, one on the 22nd and one on the 28th day of the experiment, the last count being made 11 days after the last injection in the first group and after an interval of 14 days in the second group. At this time the experiment was discontinued because the results were adequate, as shown by Tables I to V. The animals were anesthetized with ether and killed by crushing the spinal cord in the cervical region. All values represent the average of two counts made in two counting chambers. In Table V will be found the statistical data concerned with this study. The accepted criterion for observed differences to be statistically valid is that the probable error of the means must be contained at least twice and should be contained three times, for incontestable figures, in the difference between the means. The differences in the erythrocytes between the fore and after periods of the test rats and the differences between the control rats and the test rats are seen to be statistically valid.

The observations conclusively demonstrate that germanium dioxide causes a very marked increase in the number of red cells per cubic millimeter of blood. An increase in the erythrocyte count of from 1

TABLE I.

The Influence of Small Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Male Albino Rat.

Day.	Control rat.		Test rats.				GeO ₂ injected per kilo of body weight.
	R. B. C.	W. B. C.	No. 1.		No. 2.		
			R. B. C.	W. B. C.	R. B. C.	W. B. C.	
1	7.45	7.2	8.17	9.6	7.61	8.3	mg.
7	7.76	6.6	8.39	7.7	7.96	9.3	1.2*
10	—	—	—	—	—	—	1.5
14	7.57	6.5	9.26	8.6	8.42	7.9	1.8
18	—	—	—	—	—	—	2.1
21	7.78	7.1	9.60	7.8	10.37	8.6	
28	7.68	7.8	9.21	9.5	10.34	9.3	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

In Tables I to IV all values for the red cells are in terms of millions, all values for the white cells in terms of thousands.

TABLE II.

The Influence of Small Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Female Albino Rat.

Day.	Control rat.		Test rats.						GeO ₂ injected per kilo of body weight.
	R. B. C.	W. B. C.	No. 1.		No. 2.		No. 3.		
			R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
									<i>mg</i>
1	7.73	7.3	7.81	7.3	7.65	8.2	7.67	6.7	
7	7.98	7.7	7.79	7.2	7.86	8.4	7.74	7.4	1.2*
10	—	—	—	—	—	—	—	—	1.5
14	7.90	—	9.38	6.6	9.25	8.6	9.10	8.3	1.8
18	—	—	—	—	—	—	—	—	2.1
21	Killed by acci- dent.		9.68	8.7	10.09	11.3	9.60	7.5	
28			9.34	7.5	9.58	10.3	8.92	8.6	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

TABLE III.

The Influence of Large Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Male Albino Rat.

Day.	Control rat.		Test rats.						GeO ₂ injected per kilo of body weight.
	R. B. C.	W. B. C.	No. 1.		No. 2.		No. 3.		
			R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
									<i>mg.</i>
1	8.64	9.6	9.31	10.2	8.76	10.1	9.38	10.2	
7	8.61	10.4	9.30	12.2	8.70	10.1	9.38	10.1	12.0*
10	—	—	—	—	—	—	—	—	15.0
14	8.72	10.6	11.04	16.8	10.20	12.5	9.82	11.0	18.0
18	—	—	—	—	—	—	—	—	
21	8.95	9.2	10.96	12.2	10.16	8.6	10.79	9.1	
28	8.92	10.4	10.47	10.2	10.40	9.4	11.58	9.5	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

TABLE IV.

The Influence of Large Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Female Albino Rat.

Day.	Control rat.		Test rats.						GeO ₂ injected per kilo of body weight.
	R. B. C.	W. B. C.	No. 1.		No. 2.		No. 3.		
			R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
1	7.70	13.9	7.38	7.1	9.52	7.0	8.69	8.7	<i>mg.</i> 12.0* 15.0 18.0
7	7.81	17.0	7.64	11.8	9.35	10.6	8.60	8.4	
10	—	—	—	—	—	—	—	—	
14	7.97	20.0	9.20	9.4	10.54	9.1	9.36	9.6	
18	—	—	—	—	—	—	—	—	
21	8.20	18.9	9.40	12.8	11.20	12.4	10.57	9.5	
28	8.01	13.1	12.31	10.3	10.66	10.9	11.56	8.9	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

to nearly 5 million cells occurred in every one of the eleven rats which had had injections of this compound. No such response was exhibited by the control rats. Moreover, germanium dioxide does not produce a leucemia as an accompaniment of the erythrocytosis.

The combination of the fact that germanium dioxide is non-toxic and non-corrosive, with the fact that it produces such a marked increase in the number of the erythrocytes in the circulation of the healthy rat gives us the hope that this compound has a specific stimulating effect upon the erythropoietic tissue and will be found of clinical value.

An inspection of the tables will show that there is a tendency for the red count to increase most in those animals in which the original

TABLE V.
Statistical Data Concerned with the Study of the Influence of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Albino Rat.

	Control rats.				Test rats.			
	Initial counts.		Subsequent counts.		Initial counts.		Counts after GeO ₂ injections.	
	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.
Mean.	7,960,000	9,959	8,170,000	11,533	8,395,000	8,931	10,074,000	9,734
Standard deviation.	406,000	3,452	485,000	4,702	713,000	1,547	2,743,000	1,907
Probable error of mean.	97,000	823	104,000	1,057	103,000	222	322,000	223

cell count is lowest. It is also evident that the small doses cause just as marked and significant a rise as do the larger doses.

Attention should be called to the fact that there occurred a slight but statistically valid increase in the erythrocytes of the controls in each series. This increase is attributed to the possibility of food contamination through excretion of germanium, and is supported by the fact that the effect was not observed in rats used as controls in a similar series of studies with arsenic.

At autopsy marked differences were found in the color of the liver and the bone marrow of the germanium-treated rats as compared

with the controls. The liver of the control was a reddish tobacco-brown color, that of the test animals was a reddish purple. The bone marrow of the control albinos was the color of coffee with cream with here and there a slight tinge of red. That of the germanium-injected rats was maroon. No differences in the color of the spleens were detectable. A paper dealing with the histological findings will be published later.²³

SUMMARY AND CONCLUSIONS.

Injections were made of a sterile 0.4 per cent solution of germanium dioxide into four lots of mature male and female albino rats. Exact conditions of control were maintained. To two lots there was administered in four doses at intervals of 4 days a total of 6.6 mg. of the oxide per kilo of body weight. To two lots there was given in three doses at like intervals a total of 45 mg. of the compound per kilo of body weight. In each lot there was one rat which served as a control and which did not receive any germanium. A preliminary period of observation was maintained as a further control during which two determinations of the erythrocyte and leucocyte counts of the blood were made 7 days apart on all the rats. Weekly counts were made on all the rats during the progress of the investigation which lasted for 4 weeks, during 3 of which the treated animals were under the influence of germanium. In the first group 11 days elapsed between the last injection and the last count. In the second group the interval was 14 days.

It was found that without exception all of the test rats responded to the germanium dioxide by a marked and sustained rise in the number of erythrocytes in the blood which ranged from 1 to nearly 5 millions.

These results are statistically valid.

There was an apparent tendency for the degree of effect to be related to the initial erythrocyte number, in that with a lower initial count there seemed to take place a greater rise, and *vice versa*.

There was no indication that the larger doses of germanium dioxide exerted a greater stimulating effect on the production of the resultant erythrocythemia than the smaller doses.

²³ Hammett, F. S., and Nowrey, J. E., Jr., *J. Exp. Med.*, 1922, xxxv (in press).

There is evidence that the effect is quick in making its appearance. The rise in the red cell count was found to occur within a week, and after but two injections of the oxide.

Indications were obtained that germanium dioxide tends to increase the coagulability of the blood.

Autopsy findings showed color changes in the liver and bone marrow.

We believe that germanium dioxide is an erythrocytogenic agent and we hope that fruitful results will come from its therapeutic application.

THE USE OF PHENOL RED AND BROM-CRESOL PURPLE AS INDICATORS IN THE BACTERIOLOGICAL EXAMINATION OF STOOLS.

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There have been so many new media proposed during the past few years for the isolation from stools of organisms belonging to the typhoid-dysentery group that one hesitates to bring forward a new one. Believing that the indicators phenol red and brom-cresol purple possess distinct advantages over those formerly used in stool bacteriology, we have felt justified in pointing out their applicability in this particular domain of bacteriological technique.

A critical analysis of all the media advocated for the culture of stools is considered beyond the scope of this paper, but a brief mention of some of those more widely used does not seem amiss. In this country Endo's medium, prepared according to the author's formula or according to the modifications advocated by Kendall (1) or Robinson and Rettger (2), has been used perhaps more extensively than any other when a non-restraining medium was desired, although this medium has disadvantages which are well known to all workers in this field. The methylene blue-eosin agar of Holt-Harris and Teague (3) has found favor with some workers because it avoided some of the faults of the Endo medium. Meyer and Stickel (4) consider that either the medium of Holt-Harris and Teague or their own eosin-China blue medium prepared with peptic or tryptic digests is superior to the Endo agar, litmus agar, or Congo red agar "for the direct isolation and detection of dysentery bacilli from stool specimens." The extensive work of Krumwiede (5) and his coworkers upon the usefulness of brilliant green agar as a selective restraining medium has shown that this medium can be successfully applied in routine work when dealing with members of the typhoid-paratyphoid group. Teague and Clurman (6) advocate the use of a meat extract agar containing brilliant green and eosin as a selective medium for the isolation of members of the typhoid-paratyphoid group, and Meyer and Stickel¹ state that eosin and

¹ Meyer and Stickel (4), p. 67.)

brilliant green used in connection with their peptic digest agar "permits the detection of a higher percentage of viable typhoid bacilli than any other one thus far introduced into bacteriologic technic." Kligler (7) finds that the Endo medium after the addition of sulfite reaches a hydrogen ion concentration ranging from pH 8.6 to 8.8 and states that this degree of alkalinity is unfavorable to the growth of the Shiga bacillus. He also advocates the use of neutral red with the brilliant green plate of Krumwiede and considers this the best among several indicators that he tried. Kligler thinks also that the reaction of the medium has a marked influence upon the restraining activity of the brilliant green. It may be mentioned at this point that Krumwiede (8) objects to the use of eosin in connection with brilliant green because of the resultant eye strain from the irritating color produced by this combination. This objection would seem to hold good only where large numbers of plates are to be examined by one worker. Morishima (9) has recently advocated the use of a combination of phenol red and China blue as an indicator for lactose plates in the culture of stools.

In this communication it is proposed to record our experience with the use of phenolsulfonephthalein (phenol red) and dibromo-orthocresol-sulfonephthalein (brom-cresol purple) as indicators in stool bacteriology. To Clark and Lubs (10), largely, belongs the credit for the introduction of these indicators into bacteriological technique. They suggest that these indicators might be serviceable in the preparation of indicator plates (11), but so far as our survey of the literature has gone we have not been able to find any reference to the use of these indicators in stool bacteriology except in the case of the combination of phenol red with China blue, as advocated by Morishima.

Method.

In studying these indicators to determine their availability for stool work the basis of the medium used was a 1.5 and a 3 per cent beef extract agar. To this agar were added varying amounts of lactose from sterile solution and varying amounts of indicator, in order to determine the optimum amount of each of these ingredients. The reaction of the medium also was varied in order to ascertain the pH at which sharpest color differentiation was had. Plates were seeded with mixtures of *Bacillus coli* and members of the typhoid-dysentery group, also with stools inoculated with members of the latter. When the optimum amounts of the constituents had been ascertained, brilliant green was added to the agar in the proportions

recommended by Krumwiede, and the degree of restraining activity noted as compared with controls consisting of agar with brilliant green alone and agar with the indicator alone.

RESULTS.

In the case of both indicators a 3 per cent beef extract agar cleared with egg to which 1 per cent lactose had been added from sterile 20 per cent solution gave the best results. With the thicker agar there was less diffusion of acid. Thick plates, about 20 cc. of agar to a plate, were poured. We shall discuss the two indicators separately.

Phenol Red.—The optimum reaction of the agar, if this indicator is to be used, is a hydrogen ion concentration represented by a pH of 7.6 to 7.8. The optimum amount of indicator is 10 cc. of a 0.04 per cent aqueous solution for every 100 cc. of agar. The indicator may be sterilized by autoclaving. Plates poured from agar with a pH of 7.6 to 7.8 and containing the amount of indicator mentioned will assume on cooling a color best described as ranging between salmon pink and old rose. Such plates are clear, and upon them lactose-fermenting organisms produce vivid greenish yellow colonies with a surrounding zone of green. The typhoid bacillus, the paratyphoid bacillus (A and B), and the dysentery bacilli (Shiga, Flexner, and Hiss Y), all produce pink colonies. These organisms evidently produce alkali, for the surrounding medium assumes a decidedly deeper pink than the uninoculated portions of the plate. This alkali production with consequent intensification of the pink color of the medium in the neighborhood of the typhoid-dysentery colonies is a helpful factor in the differentiation of the latter. In thickly seeded plates the diffusion of the acid in the vicinity of the lactose-fermenting colonies is often sufficient to mask this change in the direction of alkalinity exhibited by the typhoid-dysentery colonies, but where good distribution is obtained the color change is most striking. Even in thickly seeded plates where typhoid and colon colonies abut upon one another, a difference in the color of the two types of colonies is observable. The colon colonies assume a bright green or yellow-green color and are opaque, whereas the typhoid colonies in such cases are more translucent and possess a bluish green color. The dye does not mask the corrugated, woolly appearance of the typhoid

colonies which is characteristic of them and upon which Krumwiede lays great stress in their recognition. On the contrary, we are inclined to feel that the dye perhaps enhances this characteristic. We have had no opportunity to study freshly isolated strains belonging to the dysentery group, and it is possible that such strains may be inhibited by this indicator; however, it does not inhibit the growth of freshly isolated strains of *Bacillus typhosus* or *paratyphosus*. The indicator itself is not reduced by the bacteria. We have found it very satisfactory when used in connection with sugar broth or Russell's double sugar.

Brom-Cresol Purple.—In our experience this indicator, when used in connection with lactose plates, gives a sharper color differentiation than does phenol red and hence is preferable to the latter. It is purple in alkaline solution, changing to yellow in the presence of acid. The color change takes place in the zone of pH 5.2 to 6.8. The optimum reaction of the agar if this indicator is to be employed is a pH of 7.2 to 7.4. The optimum amount of indicator to use is 5 to 8 cc. of a 0.04 per cent aqueous solution for every 100 cc. of agar. This solution may be sterilized in the autoclave. Plates poured from agar with a reaction of pH 7.2 to 7.4 and with the amount of indicator mentioned assume a deep blue color and are clear. Upon these plates the lactose-fermenting organisms produce greenish yellow colonies with a yellow zone, and the non-lactose fermenters produce bluish colonies. The typical appearance of the typhoid colonies is not obscured by this indicator. Even on thickly seeded plates the colonies of the non-lactose-fermenting organisms tend to preserve a bluish appearance, being bluish green rather than a brilliant yellow, the color that the colon bacillus colonies assume. It is not an infrequent experience on thickly seeded plates to see a round yellow colon colony with a wedge-shaped bluish green sector occupying a portion of the circle and representing a typhoid colony which has refused to be masked by the larger, acid-producing colony of colon bacilli. This indicator did not show any restraining influence upon any of our laboratory cultures of *Bacillus dysenteriae*, including the Shiga variety. Whether or not it would exercise any restraint on freshly isolated cultures we are not able to say.

Combination of Phenol Red or Brom-Cresol Purple with Brilliant Green.—To judge from our rather limited experience either indicator

may be used along with brilliant green in the amounts advocated by Krumwiede. We have had an opportunity to study only one sample of brilliant green, the source of which was unknown but which was active in its restraining power upon *Bacillus coli*. Neither indicator, so far as could be determined, exercised any inhibiting effect upon this restraining power of the brilliant green. The only change noticed was in the color of the plates, the phenol red-brilliant green plates being brownish red in color and the brom-cresol purple-brilliant green plates being bluish purple. The characteristic color changes produced by the lactose-fermenting colonies were not altered by the brilliant green.

DISCUSSION.

Since the introduction of lactose indicator plates for the bacteriological examination of stools for the detection of members of the typhoid-dysentery group, numerous attempts have been made to find a medium which was entirely satisfactory. As pointed out by Hiss and Zinsser (12) the best results are apt to be attained when an individual becomes thoroughly familiar with one type of medium and restricts himself to it. Endo's medium has the disadvantage of tending to vary with different batches, some being satisfactory and others useless. There can be little question but that the selective restraining media have been highly successful in skilled hands when large numbers of stools have had to be examined. Few workers, however, in carrying out routine examinations with such media, are willing entirely to dispense with a non-restraining medium such as the Endo plate, but prefer to use it in addition as a control. This is particularly apt to be the case in laboratories serving hospitals, where the proportion of stool cultures to other bacteriological examinations is not great and where the workers cannot be expected to attain the degree of skill in stool bacteriology which is attained by those carrying out an extensive series of bacteriological examinations of stools. For the less experienced worker, let us say, a non-restraining medium is essential, if only as a check, and a medium which does not obscure the characteristic appearance of the typhoid colonies and at the same time shows a sharp color differentiation should be satisfactory for the purpose.

It is because we believe that the indicators phenol red and brom-cresol purple may be successfully employed with lactose to give a

satisfactory differential plate for members of the typhoid-dysentery group when a non-restraining medium is desired and that such a medium is superior to the customary Endo plate that we have ventured to add another medium to the already long list of media proposed for the isolation of typhoid and dysentery bacilli from stools. We think the medium proposed is superior to Endo's because it gives sharper differentiation than the latter, is easier to prepare, uniform batches can be obtained, and it can be kept indefinitely if not allowed to evaporate. Of the two indicators, we prefer brom-cresol purple to phenol red.

CONCLUSIONS.

1. Either phenol red or brom-cresol purple may be used as indicators in the preparation of lactose agar plates for the isolation of members of the typhoid-dysentery group of bacteria from stools. Of the two, brom-cresol purple gives sharper differentiation and is to be preferred.

2. These indicators exercise no restraining influence upon the growth of cultures of the typhoid bacillus or paratyphoid bacillus freshly isolated from the human body, or of laboratory cultures of *Bacillus dysenteriae*.

3. Both indicators may be successfully employed with brilliant green in the isolation of the typhoid-paratyphoid group from stools without sacrificing the restraining activity of the brilliant green upon other bacteria.

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ROENTGEN RAY INTOXICATION.

I. UNIT DOSE OVER THORAX NEGATIVE—OVER ABDOMEN LETHAL.

EPITHELIUM OF SMALL INTESTINE SENSITIVE TO X-RAYS.

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The papers of this series form a part of the general program of study of the "non-specific intoxications" which is being carried forward in this laboratory. They are concerned with an investigation of the systemic intoxication which develops after a suitable exposure to large doses of hard Roentgen rays. These papers follow along the logical development of this study, based on the beginnings made by Hall and Whipple (3). Reference to the paper of Hall and Whipple is necessary for a review of some of the rather extensive literature of this subject and for a discussion of many of the fundamental problems related to Roentgen ray intoxication.

This Roentgen ray intoxication, we believe, is a true "non-specific intoxication" and deserves thorough study in part for this very reason. Too little study has been directed toward an understanding of the "non-specific intoxication" which often is a very important factor in many of the so called specific infections. This Roentgen ray intoxication closely resembles the intoxication of intestinal obstruction, another important "non-specific intoxication." We shall point out some important differences between the intoxication due to the Roentgen rays as contrasted with intestinal obstruction, but there remain many striking similarities as emphasized by Hall and Whipple (3).

We believe our experiments make it clear that the fundamental thing in the systemic intoxication due to the Roentgen rays is a *primary injury of the epithelium of the small intestine*. This was sus-

pected by Hall and Whipple but not conclusively proven. Our experiments in normal dogs under controlled conditions appear convincing when exposures given over the thorax are contrasted with the same exposures over the abdomen. For example, a unit dose (350 milliamperere minutes) given over the thorax (abdomen shielded) causes no clinical disturbance in the dog. A subsequent exposure to the same unit dose in the same dog given over the abdomen (thorax shielded) will cause fatal intoxication and death in 4 days. For a normal 30 to 40 pound dog, we may say that 350 milliamperere minutes given over the abdomen is a minimum lethal dose.

The outstanding abnormalities in the abdominal exposures are the remarkable necroses observed in the small intestine. Large areas may show complete necrosis and disappearance of the intestinal epithelium covering the villi and lining the crypts. The villi are left as naked polyps made up of stroma, vessels, and a few wandering cells. The inflammatory reaction is not as intense as would be expected under such conditions with complete removal of the covering epithelium. This important point will be discussed in detail in a subsequent paper.

Regaud, Nogier, and Lacassagne (5) described the chronic gastrointestinal lesions in dogs and noted especially gastric atrophy and intestinal perforation. Fromme (2) noted especially the clinical symptoms of gastrointestinal disturbance in guinea pigs and mice. Denis, Martin, and Aldrich (1) noted in rabbits the toxic effect of abdominal radiation and certain abnormalities in the intestines which they state are secondary to an unknown toxic factor.

Method.

The procedure is identical in all experiments, unless otherwise indicated. Normal dogs are used throughout and are kept in standard metabolism cages which have sharply pitched floors to facilitate the collection of urine. To insure the acidity of the cage collections, about 1 cc. of glacial acetic acid is added to the cage collecting bottle at the beginning of each 24 hour collection. The dogs are catheterized daily at 10 o'clock. The cage collections, cage washings, bladder washings, and catheterized urine are mixed and diluted to a unit volume—usually 2 liters. The total nitrogen is then done in dupli-

cate by the Kjeldahl method. About 300 cc. of warm water is given daily by stomach tube as a routine procedure after the dog is catheterized. Water is available at all times in the cage.

Dogs are given a subcutaneous injection of morphine shortly before exposure to radiation. A medium hard Coolidge tube is used with the target (anode or anticathode) set at a constant distance of 10 inches from the skin. At this distance the effective radiation for this machine covers a circle approximately $6\frac{1}{2}$ inches in diameter. The current strength used is either 7.5 or 8.0 milliamperes. The E.M.F. varies considerably in different experiments from 85 kilovolts to 103 kilovolts. The spark-gap backing up these voltages varies also, measuring from $8\frac{1}{2}$ to 10 inches between sharp points. The amount of radiation is calculated as the product of the current strength in milliamperes and the time of exposure in minutes and is expressed as milliamperere minutes. In all these experiments the standard autotransformer x-ray equipment of the University of California Hospital is used. Aluminum filters (2 mm.) are used to cut out the softer rays. The various parts of the dog were effectively protected from radiation, when it was so desired, by sheets of lead-foil approximately 2 mm. thick, or by lead "rubber," approximately 5 mm. in thickness.

The body of the dog is marked off roughly into two parts by a transverse line at the level of the xiphisternum. The part between this line and the base of the neck is designated as the thorax, that below as the abdomen. Each of these parts is then divided into an upper and lower half. Each upper and lower segment of the abdomen or thorax is radiated from the lateral and midventral aspects. Thus the ventral portion of the abdomen or thorax is exposed to radiation over six different areas whose contiguous edges overlap somewhat. Usually one exposure is given over the middorsal region of the thorax or abdomen, causing the total dosage to be divided up into seven parts, which, however, are spread rather diffusely over the part of the body involved. All dogs are sacrificed under chloroform or ether anesthesia by bleeding.

EXPERIMENTAL OBSERVATIONS.

Some of our typical experiments are tabulated below and make several points quite clear. However, we wish to refer to other experiments of similar nature given in subsequent papers of this series. These experiments are all in agreement and indicate that enormous doses of Roentgen rays may be given over the thorax without causing any clinical reaction in a normal dog. Dogs have been given amounts of radiation varying from 300 to 512 milliamperere minutes over the thorax without any clinical symptoms. One dog (No. 19-117) was given 350 milliamperere minutes over the thorax on three separate occasions with intervening periods of 2 to 3 weeks; all this without any apparent cumulative effects or clinical reaction. There is usually a slight transient leucopenia and there may be a very slight rise in total nitrogen excretion. After 2 or 3 weeks, the skin shows loss of hair and pigmentation as a result of the exposures. The ribs of the animal examined at autopsy show a considerable loss of marrow cell elements. We have been able to recognize no other results from these large doses of x-rays over the thorax.

Dog 19-78 (Table I) is a typical example of a complete experiment to show that a unit dose of x-rays is clinically inert over the thorax but lethal when given over the abdomen. The thorax dose causes a definite leucopenia but no clinical disturbance. That the second dose does not cause as much of a leucopenia, we believe, may be due to the tissue injury in the small intestine.

The radiation over the abdomen causes a typical clinical reaction and the review of this case will suffice for all subsequent experiments. This clinical reaction is remarkably constant and is described in detail below (Dog 19-78). The latent period of 24 to 36 hours is noted as usual and will be discussed later. The rise in total urinary nitrogen is not great but is significant. The figure for the last day includes a certain amount of fecal contamination.

Dog 19-78. Clinical History and Autopsy (See Table I).

Jan. 27. The dog is isolated on fasting diet. The animal is in good condition and quite active.

Jan. 29. The abdomen is covered with lead-foil well up to the level of the diaphragm. The thorax is exposed in seven areas, two midventral, two on each

side, and one over the midscapular region. Each area exposed covered a patch of skin approximately 5 inches square. Morphia ($\frac{3}{8}$ gr.) is given to keep the dog quiet during the exposure. The current strength is 7.5 milliamperes at an E. M. F. of 100 kilovolts with the spark-gap set at $9\frac{2}{16}$ inches. 2 mm. of aluminum filter is used and the tube is set at a target-skin distance of 10 inches.

TABLE I.

*Roentgen Radiation.**Unit Dose Given over Thorax and Subsequently over Abdomen.*

Dog 19-78. Adult, female.

Date.	Weight.	White blood cor- puscles per c. mm.	Urine.		Diet.	Remarks.
			Volume.	Total N.		
	<i>lbs.</i>		<i>cc.</i>	<i>gm.</i>		
Jan. 28	26.0	—	—	—	Water.	Good condition.
“ 29	26.0	7,600	—	—	“	
Jan. 29	X-rays, 350 milliampere minutes, given over thorax. Abdomen shielded.					
Jan. 30	25.8	8,000	—	—	Water.	Dog normal.
“ 31	24.5	4,100	—	—	“	Dog normal.
Feb. 1	23.8	4,600	—	—	“	
“ 2	23.3	4,600	—	—	“	Dog normal.
“ 3	23.0	—	—	—	Mixed.	
“ 8	27.0	5,400	—	—	“	Dog normal.
“ 21	28.3	7,400	—	—	“	
	Experiment ended.					
Mar. 15	28.9	7,800	—	—	Water.	Dog normal.
“ 17	27.8	7,600	260	2.74	“	Dog normal.
“ 18	27.3	8,000	305	2.68	“	
Mar. 18	X-rays, 350 milliampere minutes, given over abdomen. Thorax shielded.					
Mar. 19	26.8	6,400	290	2.83	Water.	Dog normal.
“ 20	26.6	7,000	265	2.74	“	Inactive.
“ 21	25.9	7,200	240	3.00	“	Vomitus and diarrhea.
“ 22	25.0	3,900	—	5.63	“	Moribund.
	Chloroform anesthesia and autopsy at once.					

Jan. 30. Dog remains normal during entire experiment (see Table I). There was a slight transient leucopenia. Dog was on mixed diet until the second experiment, also given in Table I.

Mar. 15. The dog is active and in good condition.

Mar. 18. Dog given $\frac{1}{2}$ gr. of morphia 1 hour before exposure to radiation. The thorax and head and neck are thoroughly screened with lead-foil down to the tip of the xiphisternum. Seven areas are exposed, two midventral, two on each lateral surface, and one midlumbar. The current strength is 7.5 milliamperes at an E. M. F. of 95 kilovolts, backed by a $9\frac{1}{2}$ inch spark-gap. The radiation is filtered by 2 mm. of aluminum. The skin-target distance is 10 inches. There are no immediate after effects from exposure to 350 milliampere minutes.

Mar. 19. The dog is bright and active.

Mar. 20. Slight evidences of intoxication are manifested by the dog—one soft, yellow, watery stool.

Mar. 21. The intoxication becomes marked in the afternoon. The diarrhea has progressed from brownish, watery material to tarry, black mucous or bloody mucous material with a very foul odor. Vomitus increases in amount and frequency during the day.

Mar. 22. The animal is very weak and dull. In the afternoon, the dog is completely prostrated. The stools have become very frequent and of large volume. The pulse is irregular and thready. The temperature has risen to 40.2°C . The respirations are rapid and irregular. The animal is practically moribund and is sacrificed under chloroform anesthesia.

Autopsy.—Performed immediately after death by chloroform. The hair over the thorax is thinned. It is absent over the sternum where the skin is somewhat pigmented from previous exposures to radiation. Pleural cavity, heart, and lungs are normal. Peritoneal surfaces are normal. Spleen is somewhat granular in appearance, hard and firm, normal in size. On section, the trabeculæ stand out. Mesenteric glands slightly enlarged and moist—somewhat pinkish in color. Kidneys normal in size and appearance. Bladder contracted; it shows a few ecchymoses in its mucosa (catheter). Liver is normal. Adrenals are somewhat enlarged; apparently normal except for a small necrotic mass in the left adrenal.

Stomach contains gas and about 20 cc. of a bile-stained fluid. The mucosa is pale and normal.

Duodenum is somewhat thick-walled and spastic. The lumen contains many large, round worms and a small amount of reddish-black mucus. Beginning sharply at a point approximately an inch distal to the *pyloric valve*, the mucosa of the *duodenum* presents a dark red glassy appearance. On this background run several longitudinal folds with bright red hemorrhagic crests. Numbers of small bright red petechiæ show up between these plicæ on the dark, glassy surface.

The reaction in the *jejunum* seems less intense, for here the mucosa is paler and the plicæ are not so conspicuous though they can be distinctly made out.

Ileum contains a small amount of reddish-black mucous material similar to the stools, and a few round worms. The intestinal wall is thin and rather flaccid and on close inspection the intensely hemorrhagic mucosa surface seems to have been denuded of most of the villi as the velvety appearance is lacking. The longitudinal folds with bright red hemorrhagic crests are conspicuous. Peyer's

patches seem to be stripped of their villous covering so that they show up readily as speckled oval patches. The ileocecal valve marks an abrupt end of this extensive injury.

Colon mucosa is pale, though a few longitudinal, hemorrhagic folds are present. The walls are spastic and the lumen contains a small amount of reddish fluid.

Histological Sections.

Lungs, heart, liver, pancreas, kidneys, bladder, uterus, and stomach are negative. The mesenteric lymph nodes are slightly hemorrhagic and contain some pigment. The spleen is moderately atrophic and contains pigment. In the ovary the ova are apparently dead, though nucleoli and chromatin remnants are still present within the nuclear membrane.

Pyloric duodenum: a section shows the transition from normal intact pyloric villi to collapsed and denuded villi with remnants of dead epithelium and some regenerating epithelial cells undergoing mitosis. Some areas have escaped injury.

Jejunum: destruction and disappearance of the epithelium of villi and crypts; a few crypts remain, otherwise the normal structure is gone. The epithelium around these crypts shows mitotic figures and these are apparently regeneration forms. Large pale epithelial cells stick out into the lumen of the crypts. Their cytoplasm is finely meshed and is decidedly eosinophilic. The nuclei are rather large and pale and frequently contain mitotic figures. A large amount of hemorrhage has occurred in the submucosa, and blood vessels are occasionally found plugged with thrombi of fibrin and platelets. On some areas a very thin sheet of epithelial cells can be seen making an attempt to recover the collapsed villi.

The *ileum* shows extreme injury, with the disappearance of the greater part of the crypt and villous epithelium. Strenuous attempts at regeneration are being made by remnants of the epithelium which shows many mitotic figures and is found clumped in little groups or sheets as if these groups were descendants of one or two surviving cells of the original epithelium.

The *colon* shows some mucoid degeneration and slight necrosis of the tips of a few villi, with evidence of repair.

Dog 19-85. Clinical History and Autopsy (See Tables II and IV).

Dog 19-85 received 300 milliamperes minutes over the abdomen (Table IV) which was followed by diarrhea and vomitus and prostration. She recovered. 1 month later she was given 350 milliamperes minutes over the thorax with no effect (Table II). A month later (2 months after the first exposure) she was given 350 milliamperes minutes over the abdomen with fatal result (Table II).

Mar. 2. Dog fasting since Feb. 28.

Mar. 4. Dog given $\frac{1}{4}$ gr. of morphia 1 hour before exposure to radiation. Lower part of body screened up to xiphisternum by lead-foil 2 mm. thick; 350 milliamperes minutes of radiation divided up into the usual seven areas. A cur-

rent strength of 7.5 milliamperes is used at 103 kilovolts and backed up by a $9\frac{1}{8}$ inch spark-gap. A 2 mm. aluminum filter is used. Skin-target distance 10 inches. The total time of exposure is 46.6 minutes. There are no apparent after effects from the x-ray exposure.

Mar. 5 to 8. The dog remains clinically normal.

TABLE II.

Roentgen Radiation.

Unit Dose over Thorax Negative but Subsequent Unit Dose over Abdomen Lethal.

Dog 19-85. Adult, female.

Date.	Weight.	White blood cor- puscles per c. mm.	Urine.		Diet.	Remarks.
			Volume.	Total N.		
	<i>lbs.</i>		<i>cc.</i>	<i>gm.</i>		
Mar. 2	24.7	—	—	—	Water.	Good condition.
" 3	24.4	7,800	355	2.04	"	
" 4	24.1	6,800	310	1.71		Dog normal.
Mar. 4	X-rays, 350 milliampere minutes, given over thorax. Abdomen shielded.					
Mar. 5	23.8	6,600	290	1.90	Water.	Dog normal.
" 6	23.4	8,000	325	1.79	"	" "
" 7	23.2	5,400	325	1.96	"	" "
" 8	22.9	6,800	—	—	"	
" 9	22.8	—	—	—	Mixed.	
	Experiment ended.					
Mar. 30	25.0	—	—	—	Water.	Dog normal.
" 31	24.6	—	440	2.07	"	
Apr. 1	24.1	7,400	325	1.90	"	Dog normal.
Apr. 1	X-rays, 350 milliampere minutes, given over abdomen. Thorax shielded.					
Apr. 2	23.4	7,300	300	2.13	Water.	Dog normal.
" 3	23.1	5,800	290	1.90	"	" "
" 4	22.8	7,200	240	2.07	"	Diarrhea and vomitus.
" 5	22.1	5,400	275	3.16	"	Severe intoxication.
	Ether anesthesia and autopsy at once.					

Mar. 30. Dog has remained normal since the exposure over the thorax. There is some depilation and pigmentation of previously exposed areas. Dog fasting for past 3 days.

Apr. 1. Animal given $\frac{3}{8}$ gr. of morphine 1 hour before exposure; 350 milliampere minutes of radiation spread diffusely over the abdomen in six areas, all ventral. Each area averages approximately 25 square inches. The current strength

is 7.5 milliamperes at an E. M. F. of 90 kilovolts, backed up by a 9 inch spark-gap. A 2 mm. aluminum filter is used. Skin-target distance 10 inches. There are no clinical after effects which could be attributed to the radiation.

Apr. 2 and 3. The animal is active and lively.

Apr. 4. There is quite a large volume of yellow, semifluid feces and some bile-stained vomitus throughout the morning. Intoxication becomes more marked in the afternoon. The pulse is regular, but of low tension.

Apr. 5. Greenish-brown, semifluid stools and bile-stained vomitus appear regularly throughout the day. In the afternoon, the stools are tarry and vile smelling. In general, the stools appear to contain digested or partly digested red blood cells, mixed with mucus. The vomitus at no time contains any blood, but is made up of a rather opalescent fluid containing thick, stringy mucus, which at times is bile-stained. The animal is markedly intoxicated, especially during the afternoon. Dog is able to walk but quite weak and ceases to respond voluntarily. The animal is not moribund and might survive, but in all probability would die during the night. Ether anesthesia and autopsy at once.

Autopsy.—Body fat well preserved. Pleural cavity and thoracic viscera are negative. Peritoneal cavity contains no fluid and the peritoneal surfaces are normal. Liver, spleen, pancreas, adrenals, kidneys, and genitourinary tract are negative.

Stomach contains about 20 cc. of bile-stained, frothy mucus, corresponding to the vomitus of the past 2 days. The mucosa is pale and apparently negative.

Small intestine is somewhat spastic and contains a small amount of greenish-brown mucous material.

Longitudinal folds with hemorrhagic crests are conspicuous in the duodenum. These fade out and disappear in the upper jejunum. In both the duodenum and ileum, there are patches or clusters and streaks of tiny ecchymotic specks in the mucosa. The superficial necrosis is not very extensive but stands out in contrast to the normal patches of mucosa in the jejunum and upper ileum, which are pale and velvety and apparently undisturbed.

Colon is negative.

Histological Sections.

Lungs, liver, pancreas, kidneys, adrenals, bladder, and uterus are negative. Ovary: the ova are dead, the nuclear material is broken up, and the cytoplasm is foamy. Spleen: atrophy of the Malpighian bodies and pulp elements; a few fragmented nuclei are seen. Stomach: normal. Duodenum: irregular and patchy injury of villus structures is apparent. In injured areas, there are evidences of mitosis and rapid growth of epithelial cells and some infiltration of the area by polymorphonuclear leucocytes. The irregularity of the injury is especially noted in the sections from the jejunum.

Ileum shows extensive injury and destruction of villous epithelium. The epithelium covering the villi and crypts has disappeared except for some rapidly

growing epithelial cells apparently regenerating in an effort to cover the denuded areas. Polymorphonuclear and mononuclear leucocytes are rather numerous at the bases of the crypts.

Colon shows a certain amount of mucoid degeneration and here and there an occasional focus of polymorphonuclear infiltration especially under the areas where the epithelium is injured.

Dog 19-101. Clinical History and Autopsy (See Table III).

Feb. 17. Dog is active and in good condition.

Feb. 21. $\frac{1}{4}$ gr. of morphia is given 1 hour previous to the radiation; 117 milliamper minute minutes of radiation are given with a current strength of 7.5 milliamperes,

TABLE III.

Roentgen Radiation.

350 Milliampere Minutes over Thorax Negative.

Dog 19-101.

Date.	Weight.	White blood cor- puscles per c. mm.	Urine.		Diet.	Remarks.
			Volume.	Total N.		
	<i>lbs.</i>		<i>cc.</i>	<i>gm.</i>		
Feb. 17	34.0	8,000	—	—	Water.	Dog normal.
“ 20	31.0	—	—	—		
“ 21	30.4	7,800	400	3.03		
Feb. 21	X-rays, 350 milliampere minutes, over thorax. Abdomen shielded.					
Feb. 22	30.1	7,300	415	3.02	Water.	Dog normal.
“ 23	30.0	7,900	605	2.86	“	
“ 24	29.1	5,600	565	2.69	“	
“ 25	28.8	7,300	605	2.52	“	
“ 26	28.1	7,600	408	2.49	“	
“ 27	28.1	7,400	370	2.24	“	Dog normal.
“ 28	27.8	6,900	335	2.21	“	
	Experiment ended.					
Apr. 11	31.3	7,600	—	—		
	Ether anesthesia and autopsy at once.					

at an E. M. F. of 103 kilovolts. It was then found necessary to give the remaining 233 milliampere minutes at a current strength of 10 milliamperes with an E. M. F. of 90 kilovolts. 2 mm. of aluminum filter used, spark-gap of 9 inches between sharp points. The skin-target distance is 10 inches.

Apr. 7. Dog is exposed to 450 milliampere minutes of radiation over the abdomen and sacrificed under ether anesthesia on Apr. 11.

Autopsy.—The pleural and pericardial surfaces are moist and shiny. The heart and lungs are negative. The other changes do not concern the experiment given in Table III.

Dog 19-50. Clinical History and Autopsy.

To be contrasted with Table III; a control experiment over the abdomen.

Feb. 10. Dog active and normal; fasting.

Feb. 11. $\frac{1}{4}$ gr. of morphia 1 hour previous to radiation; 340 milliampere minutes are spread over the abdomen in the usual seven areas; screening of the thorax and lower pelvis with lead-foil. A current strength of 7.5 milliamperes at 97 kilovolts is backed up by a $9\frac{1}{2}$ inch spark-gap; 2 mm. of aluminum filter the radiation. The skin-target distance is 10 inches.

Feb. 12. The dog is lively and active. A small amount of white frothy vomitus and some fluid brown feces appear on this day.

Feb. 13. There is evidence of slight intoxication. The pulse tension is low. The vomitus is of large volume, as much as a pint in an hour, and is deeply bile-stained. The diarrhea is frequent but scanty in amount and consists mostly of blood. At times, it is black, thick, and tarry. The dog is quite weak.

Feb. 14. The intoxication is quite severe. The animal is weak. The pulse is of low tension and quite irregular. The vomitus is large in amount and deeply bile-stained. The diarrhea is profuse and frequent. At times, the fecal material seems to be made up practically of pure blood and contains many clots. Again it may be tarry and vile smelling. The dog is comatose and is sacrificed under ether anesthesia. Autopsy at once.

Autopsy.—Thoracic viscera and serous surfaces are negative. The liver, spleen, adrenals, kidneys, lymph nodes, and genitourinary tract are negative. The pancreas shows a few small fat necroses.

Stomach: filled with a bloody mucous material; mucosa pale and normal.

Small intestine is spastic and contains a small amount of bloody fluid. The mucosa is a dull red, darker in the duodenum, lighter in the ileum. Longitudinal folds with hemorrhagic crests are evident in some sections, especially the paler sections of the lower jejunum, but they are masked in the duodenum by the intense dark dull red of the mucosa there. The intestinal wall is thin, apparently due to much loss of substance in the mucosa. This is especially marked in the ileum.

Colon and *cecum* contain a bloody fluid. Their mucosa is in general pale.

Histological Sections.

Liver, kidneys, adrenals, uterus are normal. Spleen shows some pulp atrophy and prominence of the reticulum. Pancreas contains a few fat necroses in one section, otherwise it is normal. In the ovary, the chromatin network of the ova is broken up, though the nucleoli are still present. The ova in general appear abnormal and seem to be degenerating.

Stomach and colon are normal.

Small intestine presents the usual picture of necrosis and destruction of villous and crypt epithelium, regeneration of epithelium with mitotic figures, and infiltration of polymorphonuclears into the bases of the crypts.

In the *ileum* the villi show only a naked reticulum with a few groups of regenerating epithelial cells and the lymph follicles. The blood vessels in the mucosa are frequently thrombosed.

The *mesenteric lymph nodes* are filled with red blood cells and polymorphonuclears and phagocytic cells containing cell debris and pigment.

TABLE IV.

*Roentgen Radiation.**Sublethal Dose over Abdomen.*

Dog 19-85.

Date.	Weight.	White blood cor- puscles per c. mm.	Diet.	Remarks.
	<i>lbs.</i>			
Feb. 4	25.0	8,000	Water.	Dog normal.
Feb. 4	X-rays, 300 milliampere minutes, over abdomen. Thorax shielded.			
Feb. 5	24.5	6,000	Water.	Dog normal.
" 6	24.0	8,000	"	
" 7	23.5	3,400	"	Inactive.
" 8	23.3	8,400	"	Vomit and diarrhea.
" 9	23.0	5,800	"	No diarrhea or vomit.
" 10	23.0	7,800	"	Clinical improvement.
" 11	22.5	5,400	Mixed.	
" 20	25.0	7,400	"	Good condition.

Dog 19-85. Clinical History (See Table IV).

Feb. 3. Dog active and in good condition; fasting.

Feb. 4. $\frac{1}{2}$ gr. of morphia given 1 hour before radiation. The upper part of the body above the level of the xiphisternum is screened by 2 mm. of sheet lead-foil. Exposures made over the abdomen and pelvis in seven parts. The current strength is 7.5 milliamperes at an E. M. F. of 100 kilovolts, backed up by a $9\frac{1}{2}$ inch spark-gap. The radiation is filtered by 2 mm. of aluminum. The skin-target distance is 10 inches. The total exposure time is 40 minutes—300 milliamperes minutes. The dog is clinically unaffected by the exposure to radiation.

Feb. 5 to 7. Dog normal.

Feb. 8. The animal shows a slight degree of intoxication. The vomitus which appears is mostly a clear frothy mucus. Diarrhea is brownish and scanty.

Feb. 9. Mild intoxication; no diarrhea or vomitus.

Feb. 10. From this date, recovery to normal is rapid.

DISCUSSION.

At the very start of this discussion, we wish to point out the fact that these experiments concern normal dogs and not human beings suffering from disease, for example leucemia, cancer, and so forth. We feel that these experiments are less complicated for this very reason and may give us the opportunity to correct mistaken impressions gotten from the complex reactions which may result from Roentgen radiation of a diseased human being.

Our negative results of thorax radiation (up to 512 milliamperes minutes) are of interest in ruling out convincingly certain of the body cells which have been suspected as causative factors in the general Roentgen ray intoxication. For example, these enormous doses given over the normal thorax must injure more bone marrow cells than do the abdomen exposures. The ribs and thoracic vertebræ surely contain more units of red marrow than do the lumbar vertebræ. Examination of the rib marrow some days after an exposure shows almost complete disappearance of red marrow cells, so we know that this reaction has taken place, yet there is not a trace of any clinical disturbance. This rules out the red marrow as a factor in the characteristic systemic disturbance noted in our experiments.

These same arguments apply to lymphatic tissue. The lymph glands in thorax and abdomen usually show some loss of cells but not to any very great extent. It is significant that Peyer's patches in the intestine may show only moderate injury when the epithelium covering the lymph follicles and lining the crypts has completely vanished due to the x-ray injury. In our experiments, therefore, the intestinal epithelium is much more sensitive to injury than are the cell elements of the lymph gland and Peyer's patch.

The spleen of the dog is not profoundly affected by the Roentgen radiation. For one thing, it is not as cellular as the human spleen, but we feel that the normal spleen cells are not easily destroyed by the x-rays. After large exposures we note a moderate atrophy of the

spleen with shrinkage of the Malpighian corpuscles, but plenty of lymphocytes remain even after intensive radiation.

The thorax exposures, of course, cover the heart and lungs, which may be dismissed without comment. This area also includes a large part of the *circulating blood*. We conclude, therefore, that the x-rays have no injurious effect upon the circulating blood cells.

The thorax exposures therefore rule out several factors which at one time or another have been thought to be responsible for the general systemic intoxication—red bone marrow, lymphatic tissue, and circulating blood. We may now consider the various tissues in the abdomen which may be responsible for the fatal reaction following large doses of the x-rays.

The kidneys are very slightly disturbed, as is shown in a paper of this series (McQuarrie and Whipple (4)).

The liver is not concerned and we believe can be ruled out without question. There is no histological evidence of injury, but more than that, there is no evidence of functional disturbance. Dogs with bile fistulæ show no disturbance in function and no change in the level of bile acid and bile pigment output.¹

The pancreas and adrenals show no histological evidence of injury by the x-rays. We know of no functional disturbance which may be attributed to these structures.

The gastrointestinal tract is remarkably sensitive to the Roentgen rays. In subsequent experiments, we shall review the remarkable ulcers which can be produced in the stomach, but as a whole, the stomach and colon are considerably more resistant than the small intestine.

The *epithelium of the small intestine* from the pylorus to the ileocecal valve is remarkably sensitive to the Roentgen rays. These doses used will cause necrosis of this epithelium with practically complete disappearance. We believe the symptoms of intoxication and the final lethal poisoning are due to this injury of the intestinal epithelium. We need not refer to the severe intoxication which develops following a functional disturbance of this same epithelium (intestinal obstruction). We may infer that the x-rays may produce sublethal injury

¹ Experiments to be published elsewhere.

to this intestinal epithelium which may be responsible for a variety of disturbances associated with gastrointestinal symptoms.

SUMMARY.

Roentgen radiation of the thorax (abdomen shielded) in dogs, even with large doses (up to 512 milliamperes minutes), gives no clinical evidence of intoxication. There may be a transient leucopenia and a slight rise in urinary nitrogen.

Roentgen radiation of the abdomen (thorax shielded) in dogs, with a dose of 350 milliamperes minutes, will almost certainly cause a fatal intoxication. Smaller doses may be survived but usually with signs of gastrointestinal intoxication.

This lethal intoxication due to abdominal radiation presents a remarkably uniform clinical and anatomical picture. There is a latent period of 24 to 36 hours, during which the dog is perfectly normal clinically. The 2nd day usually shows the beginning of diarrhea and perhaps some vomitus. The 3rd and 4th days show progressive intoxication with increasing vomiting and bloody diarrhea until the dog becomes stuporous. Death is almost always on the 4th day.

Anatomically the only lesions of significance are to be found in the small intestine. The epithelium of the crypts and villi shows more or less complete necrosis, and this condition may involve almost all of the small intestine. The epithelium may vanish completely except for a few cells here and there which have escaped and are often found in mitosis, probably an effort at repair and regeneration.

We are forced to the conclusion that this remarkable injury of the epithelium of the small intestine is responsible for the various abnormal reactions and final lethal intoxication which follow a unit dose of Roentgen radiation over the abdomen of a normal dog. This sensitiveness of the intestinal epithelium to x-rays is not appreciated and should be given proper consideration in clinical work.

In conclusion we wish to express our sincere appreciation for the assistance and advice given us by members of the Department of Roentgenology of the University of California Hospital—Drs. Howard E. Ruggles, Lloyd Bryan, Orrin S. Cook, and Elizabeth Schulze.

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ROENTGEN RAY INTOXICATION.

II. A STUDY OF THE SEQUENCE OF CLINICAL, ANATOMICAL, AND HISTOLOGICAL CHANGES FOLLOWING A UNIT DOSE OF X-RAYS.

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The experimental data given below supplement that of the first paper of this series. All these experiments make it very clear that the initial injury of the epithelium of the small intestine is responsible for the severe clinical reaction and lethal intoxication. The fact that this epithelium is sensitive to the Roentgen rays has not hitherto been clearly recognized. For this reason, it seemed desirable to study carefully this reaction and record the histological and gross changes which follow day by day after the initial exposure.

It is well to recall that the urinary nitrogen in these experiments shows a prompt increase on the day following the exposure (experiments in Paper I and those of Hall and Whipple (2)). This increase is not great and no considerable rise is usually noted until the last day before death. This urinary nitrogen curve does not in any way parallel the clinical reaction, which usually presents a latent period of 24 to 48 hours. It is of great interest to note below that within the first 24 hours, there are distinct histological abnormalities in the epithelium of the small intestine, yet this is always a period free from any clinical evidence of injury but showing a slight rise in urinary nitrogen.

The appearance of clinical intoxication coincides with the actual initial cell disintegration which is so evident in the epithelium of the small intestine in the second 24 hours. It is of interest that the crypt epithelium suffers first and shows evidence of actual necrosis and solution before any change is apparent in the epithelium covering the villi. This may well indicate some undetermined differences in

function as well as in histology between these two types of epithelial cell. The epithelium of the villi does not show much disturbance until the 3rd day, when the clinical intoxication is also well marked with bloody diarrhea and bile-stained vomitus.

The severity of the intoxication on the 3rd and 4th days coincides with the disappearance of much of the epithelium all through the small intestine. One may choose to explain this reaction as due to absorption of toxic substances and *invasion of bacteria from the lumen of the injured intestine*. These factors may be in part responsible for the intoxication, but we believe there are other possibilities which must be considered. Some of these are to be reviewed more in detail in subsequent papers of this series.

EXPERIMENTAL OBSERVATIONS.

All materials used in this study are obtained from dogs killed under anesthesia and autopsied at once. Tissues are fixed at once in formaldehyde (10 per cent) and stained usually by the routine hematoxylin-eosin method. Control tissues from normal dogs are fixed and prepared in exactly the same manner. A minimum of mechanical handling before fixation is necessary in this work. Routine examination of all organs in gross and histologically is carefully performed, but in the protocols normal findings are often omitted from the record.

Experiment 1. Lethal Dose of Roentgen Rays over Abdomen. Killed after 2 Hours.

Dog 20-33.—Normal, mongrel, adult, male; weight 32.5 pounds. Dog fasting for 4 days previous to radiation. Roentgen ray exposure over abdomen in four quadrants; no screens used, but usual 2 mm. aluminum filter. In all, 480 milliamperes minutes given; E. M. F. of 89 kilovolts; current strength 8 milliamperes; skin-target distance of 10 inches. Exposure given between 3 and 5 p. m. 6 p. m. Dog sacrificed by bleeding under ether anesthesia.

Autopsy.—Performed at once. All viscera examined carefully and found to be normal, except for the kidneys, which show a few scars in the cortex. The intestinal tract shows no congestion and no abnormalities of any sort.

Histological Sections.—All organs and tissues examined in routine sections. There are very few abnormalities. The kidneys show arteriosclerosis and dense scars; the prostate shows some hyperplasia. Spleen and mesenteric glands show a good deal of nuclear fragmentation in centers of follicles.

Gastrointestinal tract at first sight is negative, but careful comparison with sections of normal control dogs convinces one that some of the nuclei in the crypt epithelium of the duodenum and jejunum do show slight abnormalities. These nuclei are fuzzy looking and the chromatin is beginning to show very slight evidence of injury. These changes are much more noticeable in the lymph glands of this region.

Experiment 2. Lethal Dose of Roentgen Rays over Abdomen. Killed after 24 Hours.

Dog 19-130.—Apr. 15, 2 p.m. Normal, old adult, male mongrel; weight 45 pounds. Dog fasting for 3 days previous to radiation. X-ray exposure over abdomen, 450 milliamperere minutes; 90 kilovolts; current strength 7.5 milliamperes; spark-gap 9 inches; 2 mm. aluminum filter; skin-target distance 10 inches. Dog given morphia ($\frac{1}{2}$ gr.) before exposure.

Apr. 16, 2 p. m. Dog normal in all respects; chloroform anesthesia and exsanguination.

Autopsy.—Done at once. All viscera examined with care and are normal with the following exceptions: *Small intestine* is tightly contracted and contains only bile-stained fluid. Duodenum and jejunum show the mucosa thrown up into longitudinal folds whose crests are specked with ecchymoses. There are a few small scattered ecchymoses all through the small intestine. The mucosa is not markedly congested but is unusually *bile-stained* and this may be due to actual vital staining of the injured cells.

Histological Sections.—Spleen and mesenteric lymph nodes show cell necrosis in lymph follicles and numbers of phagocytes. All of small intestine shows definite nuclear changes in the epithelium of the crypts. Nuclei are pale or show fragmented chromatin material. There is a definite invasion of polymorphonuclear leucocytes. The epithelium of the tips of the villi shows no distinct change as yet.

Experiment 3. Lethal Dose of Roentgen Rays over Abdomen. Killed after 48 Hours.

Dog 19-101.—Apr. 9. Normal, adult, female mongrel; weight 33 pounds. Dog fasting for 4 days and through experiment. X-ray exposure over abdomen, 450 milliamperere minutes; 90 kilovolts; current strength 7.5 milliamperes; spark-gap 9 inches; 2 mm. aluminum filter; skin-target distance 10 inches. Dog given morphia ($\frac{1}{2}$ gr.) before exposure. Thorax and pelvis screened by lead.

Apr. 10. Dog is normal.

Apr. 11. Dog bright and active, but vomits some bile-stained mucus at 2 p. m. Ether anesthesia and sacrifice at 4 p. m.

Autopsy.—At once. All organs examined with care and are normal except where noted otherwise. Stomach contains a little bile-stained mucus, but mucosa is normal. Small intestine contains a little bile-stained mucus and a little bright

red blood in one section; the mucosa in general is pale, but shows scattered ecchymoses. There are longitudinal folds whose crests are pink and dotted with ecchymoses. There are few congested patches in the ileum. Colon is spastic but mucosa normal.

Histological Sections.—Stomach mucosa is normal. There is a sharp line of transition from normal stomach to abnormal duodenal mucosa. Here the duodenal crypts show fragmented nuclei and cell degeneration. A few wandering cells are in evidence. Mitotic figures indicate an effort on the part of the epithelium to regenerate. There is some edema of the submucous tissues. The epithelium covering the villi appears uninjured. This condition is general throughout the small intestine. There is a sharp line of demarcation at the ileocecal valve and the colon mucosa seems practically normal. Colon shows occasional crypts presenting degenerating epithelial cells and invasion by wandering cells. Ovary shows degenerating ova with chromatin fragmentation.

Experiment 4. Lethal Dose of Roentgen Rays over Abdomen. Killed after 3 Days.

Dog 19-50.—Feb. 11. Normal, adult, female spaniel; weight 32 pounds. Dog fasting for 2 days before radiation and through experiment. X-ray exposure over abdomen, 340 milliamperere minutes; 97 kilovolts; current strength 7.5 milliamperes; spark-gap $9\frac{1}{2}$ inches; 2 mm. aluminum filter; skin-target distance 10 inches. Thorax and pelvis shielded by lead. Morphia ($\frac{1}{4}$ gr.) before exposure.

Feb. 12. Dog lively; a little frothy vomitus in afternoon.

Feb. 13. Dog less active; blood-stained stools and bile-stained vomitus.

Feb. 14. Dog weak, much bright blood in stools, which contain mucus and clots, very foul smelling; vomiting less frequent. Ether anesthesia and sacrifice.

Autopsy.—At once. Viscera all examined carefully and found normal unless otherwise noted. Kidney: slight scarring of cortex. Pancreas: a few tiny fat necroses close to bile duct. Stomach contains blood-tinged, bile-stained mucus, but its mucosa seems normal. Small intestine contains a little bloody mucus and it is irregularly contracted. *Duodenum* shows a dark, glassy, red mucosa which is thin and in places wrinkled. The mucosa has lost its velvety appearance. This is explained by the histology. The rest of the small intestine shows much the same picture but the surface is mottled and some areas are much more abnormal than others. Peyer's patches are rather conspicuous, due to the thinning of the mucosa. This change in the mucosa begins sharply at the pylorus and ends as sharply at the ileocecal valve. Colon: mucosa is in general pale but there are a few ecchymoses.

Histological Sections.—Unless noted are negative. Lymph follicles in mesenteric glands and spleen show evidence of injury and removal of damaged cells. Ovary shows degenerating and necrotic ova. Bone marrow—lumbar vertebra—shows few marrow cells remaining and much cell debris in large phagocytes. Stomach: some cells in the mucosa appear slightly abnormal but there is nothing definite. *Duodenum*: much of the epithelium covering the villi and lining the

crypts has disappeared. In many places, the villi appear as naked polyps, a little swollen and containing dilated capillaries. The crypts are often filled with cell debris and wandering cells. At times mitotic figures are seen in the remaining epithelium. Wandering cells are conspicuous in all this area—usually polymorphonuclears. There is a noticeable edema of the submucosa. The whole structure of the mucosa has collapsed because of the destruction of the epithelium. Some sections show a mucosa which is almost normal. This "escape" of these areas will be discussed later. *Jejunum* and *ileum* show the same extensive destruction of the epithelium with consequent collapse of the familiar structure of the mucosa. Many sections would scarcely be recognized as coming from the small intestine. In places the mucous surface is covered with an exudate of red and white cells with fibrin. Some areas show only a moderate injury, corresponding to the gross appearance.

Experiment 5. Lethal Dose of Roentgen Rays over Abdomen. Killed after 4 Days.

Dog 19-80.—Jan. 21. Adult, female mongrel; weight 20.5 pounds. Dog fasting for 2 days previous to radiation and subsequently. X-ray exposure over abdomen, 350 milliamperes minutes; 95 kilovolts; current strength 7.5 milliamperes; spark-gap $9\frac{1}{2}$ inches; skin-target distance 10 inches; 2 mm. aluminum filter. Dog given morphia ($\frac{1}{4}$ gr.) before radiation. Thorax and pelvis screened with lead.

Jan. 22. Dog active and normal.

Jan. 23. Dog less active, but no other clinical symptoms.

Jan. 24. Dog sick with soft stools and usual vomitus.

Jan. 25. Dog prostrated; stools contain fresh blood and clots; temperature is falling and dog becoming comatose; ether anesthesia and sacrifice 90 hours after radiation.

Autopsy.—At once. Unless otherwise stated viscera are normal. Mesenteric glands are enlarged, moist, and congested. Stomach contains blood-tinged mucus and its mucosa is normal. Small intestine contains a little bloody fluid and a few worms. The mucosa in many loops is dark red and glassy, specked with ecchymoses. Other areas are more nearly normal and show the velvety texture of the mucosa. The jejunum seems to be less uniformly injured than the duodenum and ileum. Longitudinal folds with hemorrhagic crests are found in all parts of the small intestine. Colon shows a pale mucosa specked with occasional ecchymoses.

Histological Sections.—The changes noted in this autopsy are almost identical with those described in detail in Experiment 4. The epithelial cells of the mucosa are conspicuous by their absence, and there is more evidence of mitosis and early repair on the part of the few epithelial cells which remain in the injured crypts. There is a moderate grade of edema of the submucosa and some invasion by wandering cells. The congested capillaries in the naked villi are the obvious

source of the blood in the intestinal contents. Occasional thrombi are noted in these vessels. Stomach and colon show minimal changes. In similar experiments one may find considerable epithelial degeneration in the crypts of the colon mucosa but it is obvious that this epithelium is much more resistant than is that of the ileum.

DISCUSSION.

We may review the positive features of this Roentgen ray reaction in dogs day by day following the exposure. The first 24 hours show no clinical disturbance. These dogs would pass as normal on the most critical examination. The urinary nitrogen shows a slight but constant increase above normal. The body cells, however, show some definite changes. The spleen and lymph glands show readily recognized cell injury and nuclear fragmentation within 2 hours. The epithelium of the crypts of the small intestine also within 2 hours shows distinct but faint nuclear changes. Within 24 hours, this intestinal epithelium shows nuclear fragmentation and cell injury together with a wandering cell reaction (leucocytes and macrophages). The ova in the ovary show the familiar nuclear reaction of degeneration.

After 2 hours, the small intestine may show a distinct flushing of the mucosa. This may or may not be due wholly to the x-rays, but it may be noted as perhaps similar to the erythema of the skin which may be noted a few hours after an erythema skin dose. The skin may not show distinct injury (burn) for many days after this initial reaction to injury by the x-rays. Apparently the various body cells have individual peculiarities in their reaction to toxic doses of the x-rays, especially as regards the latent period. Compare leucocytes, intestinal epithelium, and skin epithelium.

Within the second 24 hours after a toxic dose of the x-rays, we see many distinct changes from normal. Usually there is definite clinical abnormality, although at times this may be very slight. As a rule, there is loss of appetite and activity. The dog often vomits a little mucus and passes a soft stool or two. Autopsy at this time shows an intestinal tract which may appear normal except for a few ecchymoses in the small intestine. There may be abnormal bile staining of the mucosa, due in part perhaps to the presence of dead epithelium. The intestine in general is apt to be spastic and reacts to the stimuli with very vigorous contractions. This irritation of the muscle coats

is probably the result of the early inflammation beginning in the mucosa and submucosa. This spastic tendency, we believe, explains the longitudinal folds which are so conspicuous in the 3rd and 4th days with their crests marked out by congestion and hemorrhage.

Histologically, in the second 24 hours, we find a number of important changes, practically all in the small intestine. The epithelium of the crypts shows severe injury with nuclear destruction and even cell necrosis and desquamation, so that the crypt lumina are filled with cell debris and wandering cells. The epithelium of the villi still remains to all intents normal. The wandering cells are not yet conspicuous in the submucosa and are usually polymorphonuclears. There are a little submucous edema and occasional extravasation of red cells. These changes are conspicuous from the pylorus to the ileocecal valve. The colon shows occasional small patches in which the crypts show similar but less advanced injury.

The third 24 hour period is one of violent clinical disturbance and frank anatomical changes, mostly limited to the small intestine. The dog is constantly retching and frequently vomiting a thick mucoid bile-stained material. There is usually much fluid diarrhea, often with blood and mucus. Tenesmus may be much in evidence. Smears from fecal material show numbers of red and white blood cells, epithelial cells, and bacteria. This period gives marked anatomical changes in a typical case. The entire small intestine from pylorus to ileocecal valve looks raw, red, and thinned out. Some areas may escape and appear relatively normal. We believe these areas of severe injury of the mucosa are to be explained by overlapping areas of exposure. They may also be in the uppermost coils of intestine in the abdomen.

The histological material is of great interest but we cannot review the details. There is almost complete loss of crypt and villous epithelium, and the mucosa collapses into an atypical matrix of villi and empty crypts. Some sections are almost unrecognizable. Villi appear as naked polyps with congested capillaries. A few crypts may contain occasional epithelial cells which are large and contain mitotic figures—an attempt at early repair. The bloody fecal contents are easily explained by this picture. In spite of these raw areas we do not observe the intense inflammatory reaction which might be

expected. There are a little edema and a distinct increase in wandering cells. We believe this point is of much interest as regards the common conception of the intestinal epithelium as the sole barrier between the intestinal bacteria and the blood and lymph stream. This point will be taken up again in detail with a study of its bacteriology.

The fourth 24 hour period is much like the third but shows increasing intoxication. Death usually follows on this day. There is great loss of weight and strength. Vomitus and diarrhea continue unabated and the dog finally goes into coma preceding death. The histology is much like that described for the 3rd day, but evidences of epithelial repair may be more conspicuous and of fascinating interest to the histologist.

It is a common observation that lymph follicles and Peyer's patches show less injury than do the neighboring epithelial cells of the mucosa. Our sections would indicate that the epithelium of the small intestine is at least as sensitive to the x-rays as are the lymphocytes of the same area. In fact, by a large dose of the x-rays, we can actually destroy all of the epithelium, while many clusters of lymphocytes still remain. It is possible, of course, that lymphocytes in different parts of the body have varying susceptibilities to radiation, so that this observation in the intestine may not be applicable to the lymphocytes elsewhere in the body.

It may be noted at this time that a recent publication of Denis, Martin, and Aldrich (1) differs considerably in its interpretation of the abnormalities noted after long x-ray exposures. Rabbits were used and in our experience are not as favorable animals for this work, although the changes are similar. In their discussion, Denis, Martin, and Aldrich suggest that these epithelial changes in the intestine are "the result rather than the cause of the intoxication" and not primarily responsible for the intoxication. We are confident that our experiments give convincing proof that this epithelial injury in the small intestine is due to the x-rays and is in itself responsible for the progressive and lethal intoxication.

SUMMARY.

Roentgen radiation in lethal dosage, given over the abdomen of a normal dog, is followed by a physiological reaction of remarkable uniformity.

The first 24 hour period following the exposure is negative clinically and anatomically, but histologically we see frank changes in the bone marrow, spleen, lymph glands, and ovaries. There are definite nuclear changes with degeneration in the crypt epithelium of the small intestine.

The second 24 hour period shows slight clinical disturbances of gastrointestinal nature (vomitus and diarrhea). The mucosa of the small intestine shows scattered ecchymoses, but the histology of the small intestine is important. The necrosis of the crypt epithelium may be almost complete, while the epithelium of the villi remains practically intact. There are a little edema and invasion of wandering cells.

The third 24 hour period shows increasing clinical disturbance with vomiting and bloody diarrhea. Anatomically the small intestine from the edge of the pylorus to the rim of the ileocecal valve looks raw, red, and inflamed. The crypt and villous epithelium has in large part vanished, leaving a collapsed framework of the mucosa showing a little edema and invasion of wandering cells.

The 4th day marks the peak of the intoxication, and death usually takes place at this time, preceded by coma. Anatomically and histologically the picture is like that of the 3rd day. There is more evidence of mitosis and efforts of repair on the part of the intestinal epithelium.

The stomach is not concerned in this reaction, but the colon may show evidences of a slight injury. The colon is obviously much more resistant than is the small intestine.

We believe the evidence is conclusive that the injury done the epithelium of the small intestine is wholly responsible for the stormy clinical picture and fatal intoxication.

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ROENTGEN RAY INTOXICATION.

III. SPEED OF AUTOLYSIS OF VARIOUS BODY TISSUES AFTER LETHAL X-RAY EXPOSURES.

THE REMARKABLE DISTURBANCE IN THE EPITHELIUM OF THE SMALL INTESTINE.

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The question of Roentgen ray injury and certain modifications of intracellular ferments has been reviewed by Hall and Whipple (1). We must admit that the various methods of studying intracellular ferments are at present very crude and probably give mere hints as to what goes on within the cell. These ferments are accustomed to their normal environment which is very carefully balanced by the presence of the circulating fluids which remove waste products and furnish necessary supplies to the cells. The reaction of these ferments in a mass of dead or dying cells cut off from circulatory influences may or may not have any significance as to the true physiological function of these ferments.

We have submitted much evidence of true cell injury involving both nucleus and protoplasm following x-ray exposures. We submit below evidence to show that cell autolysis in the epithelium of the small intestine is profoundly modified by x-ray exposures. Some investigators go so far as to claim that primary injury of cell ferments is a fact and is in itself responsible for subsequent reactions and cell death. But how can one tell that this modification of cell autolysis is due to a primary ferment influence or some other physiological disturbance which influences the ferments secondarily? When the investigator gives this explanation of primary cell ferment reaction, he is indeed in distress when he attempts to explain the great differ-

ences in the effect of the x-rays on different cell structures. Our methods permit of a distinction in cell and nuclear structure between these cells which react so very differently to the x-rays. But present methods do not permit us to recognize a great multitude of individual cell ferments in the parenchymatous organ cells. Compare the cells of the bone marrow, intestinal mucosa, pancreas, and liver. They are all rich in ferments but they react very differently indeed to the x-rays. One must be very conservative in the analysis of data concerning intracellular ferments and deductions drawn from such experiments.

It was pointed out by Hall and Whipple (1) that the intestinal epithelium in radiated dogs was subject to rapid postmortem autolysis, so much so, that a few hours after the death of the radiated animal very little of the epithelium could be demonstrated. These workers also indicated that the crypt epithelium was more abnormal in this respect than the tip epithelium. With these observations in mind, a series of autolysis experiments was undertaken using sections of organs from the series of dogs described in Paper II. In this series an attempt is made to demonstrate the various changes in cell autolysis observed at various periods following a lethal x-ray exposure.

Method.

A series of dogs was exposed to 350 to 480 milliamperes minutes of radiation diffusely over the abdomen. These experiments are described in the preceding paper. Dogs were sacrificed under chloroform or ether anesthesia at various periods of 2, 24, 48, 72, or 96 hours after exposure to radiation. A set of sections of each organ for control is fixed immediately in 10 per cent formaldehyde. Five sets of sections, each section about 3 to 5 gm. in weight, are cut from each organ immediately after death of the animal. Each section is placed, while still warm, in a bottle containing approximately 150 cc. of warm 0.9 per cent sodium chloride solution previously saturated while warm by shaking for an hour with an excess of chloroform. All the bottles are then warmed to 37°C., corked, placed in an incubator at 37°C., and the tissues allowed to autolyze. A set of sections is removed at 4, 8, 10, 12, and 24 hours respectively and immediately fixed in 10 per cent formaldehyde. There is no putrefactive odor

present in the autolysis bottles, even after 24 hours autolysis of intestinal sections, indicating that the growth of putrefactive bacteria has been inhibited by the chloroform and suggesting that the autolysis is due to the endogenous ferments. There are no free chloroform globules present in the autolysis bottles, it being completely held in solution. The odor of chloroform is still quite strong after 24 hours autolysis. Several sets of experiments were done with sodium chloride solution without chloroform, but these were found to be very irregular, probably due to variation in the outgrowth of bacteria.

Organs from three normal unirradiated dogs are treated to the same routine and used as controls.

Histological Method.

The autolysis sections are studied with regard to the effect of radiation upon the rapidity of autolysis *in vitro*. Gradations of autolysis are based upon the intensity of staining of the nuclei, the integrity of the cell outline and the cell contents, and the complete digestion of the cell itself. In designating changes for the purposes of constructing curves indicating the speed of autolysis, the following criteria are used:

0 means no autolysis perceptible.

A or + means cell nuclei are pale and washed out, as compared with the control of that organ—slight autolysis.

B or ++ means nuclear structure is lost, run together, or indistinct—well advanced autolysis.

C or +++ means the nuclear outline is shadowy and indistinct. There may appear to be nothing left but nuclear debris in the space occupied by the cell—marked autolysis.

D or T means total autolysis; the nuclei are gone, though the basement membranes are usually intact. At this stage, there is nothing left but pale fibroblasts, polymorphonuclears, endothelial cells of the blood vessels, and the fibrillar intracellular matrix.

It would seem that these arbitrary periods of autolysis are irregular in sequence but these periods were established by preliminary experiments and seemed to us to give the required information. In plotting these findings, it also serves our purpose to consider the individual stages (or extent) of autolysis and the individual period of

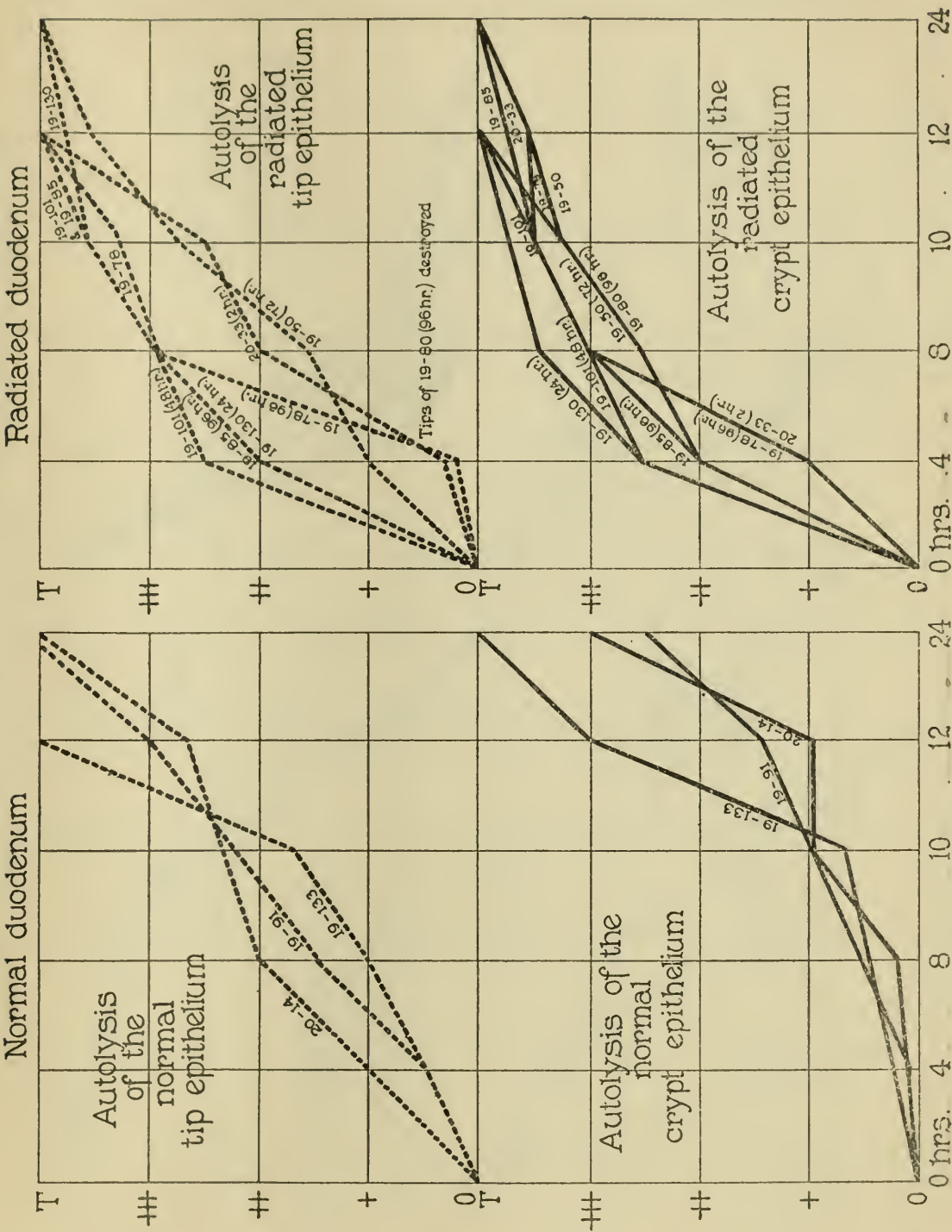
autolysis as equal and numerically progressing units upon the coordinates. It is evident that if all of the constants are equal and presented in the same manner, the experimental results are being compared upon a satisfactory basis. Therefore, although all of our units are arbitrary, they are constant throughout and any consistent variation in the plotted curves may be considered as demonstrating changes produced by the experimental procedure (exposure to radiation).

EXPERIMENTAL OBSERVATIONS.

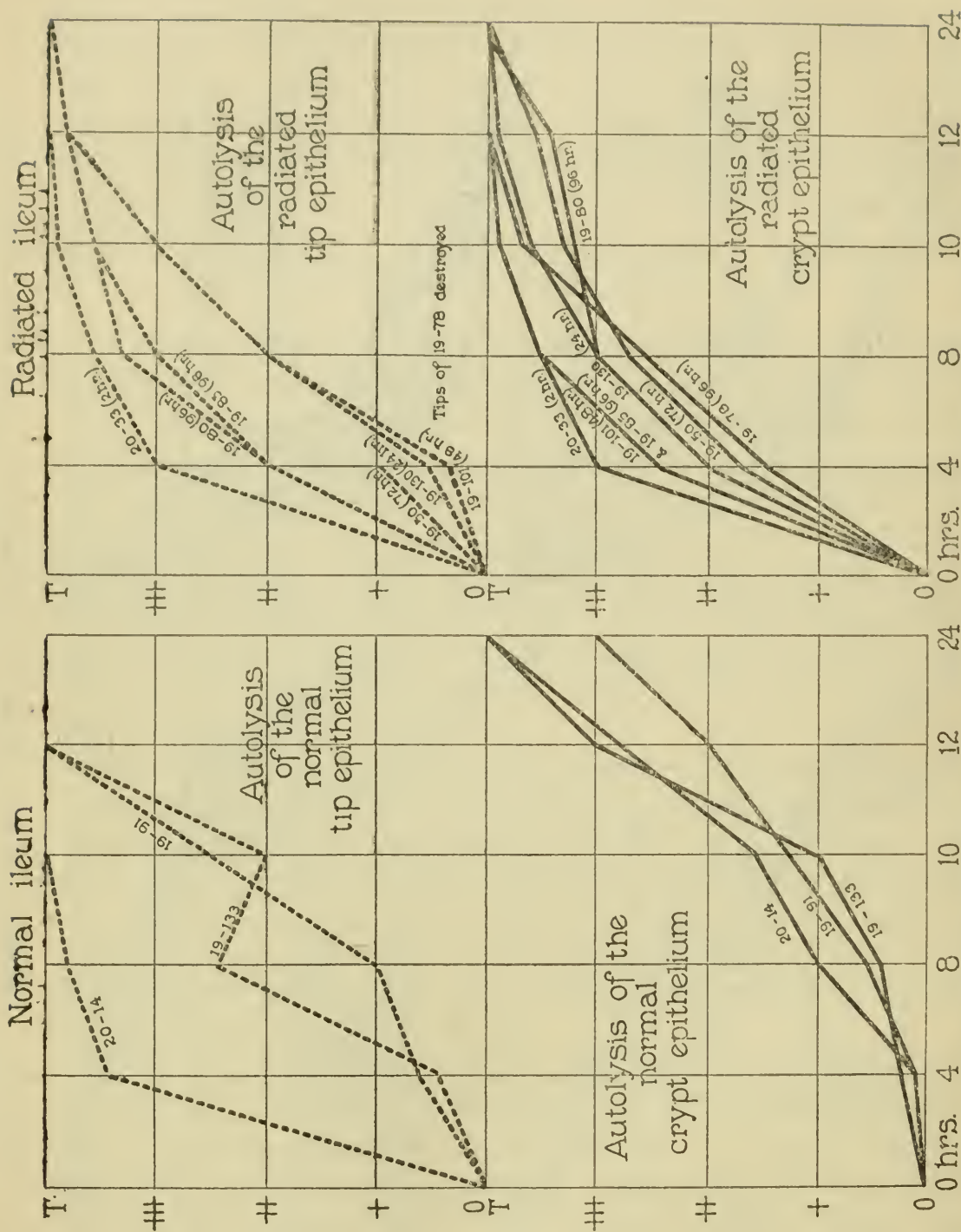
All the histological material for this study was fixed in 10 per cent formaldehyde and stained by the usual hematoxylin-eosin method, using paraffin sections. About 600 slides were studied and reviewed at least three separate times as unknown specimens to prevent any bias from knowledge of sequence of events in autolysis periods. The grades of autolysis have been established and described in the preceding paragraph. Curves of the various tissue reactions were established in form similar to Text-figs. 1 and 2. These many charts cannot be published but the statements made below are based on a study of such charts and sections. Much of the more important data can be studied in Table I.

We may note that certain cells common to organs and tissues are not much, if at all, disturbed by these long x-ray exposures. Such are fibroblasts and mature connective tissue cells, endothelial cells and macrophages, muscle tissues, and certain white blood cells. All these tissues are resistant to autolysis in the control and radiated animals and may appear as very little changed even by the long 24 hour period of autolysis at 37°C. Some, but not all, of these cells are supposed to be poor in ferments.

The more specialized cell types of the abdominal viscera are our chief concern. In the gastrointestinal tract, we differentiate between the tip epithelium which covers the villus and the crypt epithelium which is more of the secreting cell type. In the spleen and mesenteric lymph nodes, we have made a rather broad distinction between the pulp and the Malpighian bodies; in the pancreas between the isles of Langerhans and the remainder of the parenchyma. In the kidney, the proximal convoluted tubules are contrasted with the straight



TEXT-FIG. 1. Comparison of the autolysis of the duodenum of normal and of radiated dogs.



TEXT-FIG. 2. Comparison of the autolysis of the ileum of normal and of radiated dogs.

TABLE I.
Autolysis during Periods of Hours as Indicated.

Hrs. autolysis.	Control.				2 hrs. after x-rays.				24 hrs. after x-rays.				48 hrs. after x-rays.				72 hrs. after x-rays.			
	4	8	10	12	24	4	8	10	12	24	4	8	10	12	24	4	8	10	12	24
Duodenum crypt.....	0	0	A	A	C	A	C	C	D	—	B	C	D	D	—	B	C	D	D	—
“ villi.....	A	A	C	C	D	“	B	“	C	D	“	“	“	“	—	A	B	C	“	—
Jejunum crypt.....	0	0	A	B	C	B	C	D	“	—	“	“	B	C	D	“	C	“	“	D
“ villi.....	0	A	B	C	D	0	A	B	C	D	A	“	C	D	—	—	—	D	—	—
Ileum crypt.....	0	“	A	B	C	C	C	D	“	—	B	“	D	C	—	A	C	D	—	—
“ villi.....	A	B	B	C	D	B	“	“	“	—	A	B	C	“	—	“	—	—	D	—
Colon.....	0	A	“	“	“	A	B	C	“	—	B	C	“	“	—	“	B	C	D	D
Stomach.....	A	“	“	“	“	B	C	D	“	—	A	“	B	“	D	“	“	“	“	“
Liver.....	“	B	“	“	“	“	“	C	“	—	“	C	“	“	—	“	“	“	“	“
Pancreas.....	0	A	“	“	“	“	“	“	“	—	B	“	“	“	D	“	“	A	B	“
Spleen.....	A	B	“	“	“	“	“	“	“	—	C	“	C	D	“	“	“	B	“	“
Kidney.....	0	A	“	“	C	A	B	B	A	C	0	A	A	C	C	B	“	C	C	“

Degree of autolysis indicated as follows (see complete description under Histological method): 0 means zero autolysis. A means slight autolysis. B means well advanced autolysis. C means marked autolysis. D means total autolysis.

tubules and the glomeruli, mainly because of their difference in autolysis; in the liver, the parenchyma cells are contrasted with the bile ducts.

In the normal intestine, the autolytic digestion of the mucosa starts in the tips of the villi and progresses fairly slowly and evenly to the bases of the crypts. Even at the end of 24 hours, the crypt epithelium may be fairly well preserved while the tip epithelium is almost totally autolyzed. In the *radiated animal*, on the other hand, *the crypt epithelium autolyzes at a greater speed than the tip epithelium* and is practically completely autolyzed in 10 to 12 hours or earlier, while even after 12 hours of autolysis the tip epithelium in the same animal may be fairly well preserved (compare Text-figs. 1 and 2).

Discussion of Experiments.

Normal Autolysis.—Three normal dogs are sacrificed under anesthesia. Sections of abdominal viscera are autolyzed at body temperature in 0.9 per cent sodium chloride solution saturated while warm with chloroform as described above. The usual course of digestion is from without inward and the progress is steady and continuous. The digestion in the gastrointestinal sections progresses from the villi to the bases of the crypts. Comparisons are made of the penetration of the autolysis as well as of the progress of cell digestion.

Stomach: The chief and parietal cells of the gastric mucosa are more susceptible to autolysis than are the tip cells. The former are autolyzed rapidly and are totally digested at the end of 10 hours. The tip cells autolyze somewhat more slowly and are completely digested in 24 hours.

Duodenum, jejunum, ileum, and colon are quite similar in the type of their autolysis. The tip epithelium is the first to autolyze. In these control experiments the time may vary from 10 to 24 hours before completion of the digestion. Considerable variation is to be noted in this reaction of the epithelium of the villi. The autolysis of the crypts is usually delayed 4 to 8 hours, after which it proceeds regularly though usually not to completion in 24 hours (Text-figs. 1 and 2).

Spleen pulp is found to autolyze 4 to 8 hours faster than the Malpighian bodies, which, as a rule, are not completely autolyzed by 24 hours. Their centers are fairly well preserved at 12 hours. The pulp at this time is usually pretty well digested.

Pancreas digests pretty well as a unit. The islands of Langerhans autolyze on the average 2 to 4 hours more slowly at first than the parenchyma but later the speed varies considerably so that they may totally digest in 10 hours, or only partly digest in the full 24 hours.

Kidney convoluted tubules are very sensitive to autolysis. Usually they are completely digested in 8 or 10 hours. The remainder of the *kidney parenchyma* consisting of the straight and collecting tubules and the glomeruli is more slowly digested, the full 24 hours usually being required for their complete autolysis.

Liver parenchyma and bile capillaries autolyze as a unit.

Autolysis of Radiated Organs.—Seven normal dogs are exposed to large amounts of radiation, given diffusely over the abdomen, and sacrificed at definite periods after exposure. Autolysis specimens are studied (as above) in regard to the progress of digestion in the specialized cells of the various organs.

Stomach epithelium, though the speed of autolysis may seem to have increased slightly, is digested well within normal limits. This is true for the whole series of dogs which were killed 2, 24, 48, 72, and 96 hours after radiation.

Intestinal epithelium shows a striking change from the controls. The speed of autolysis of the tip epithelium in each case is practically within normal limits. The great change has occurred in the speed of the autolysis of the crypt epithelium. The curves in Text-figs. 1 and 2 illustrate this very well.

The speed of autolysis of the crypt epithelium is rapid and must be due to the immediate effect of radiation upon these cells. The most pronounced increase in the speed of autolysis occurs within the first 2 hours after exposure to radiation. The change produced in these cells, or what is left of them, is permanent up to the time of the death of the animal 4 days later. Even after a lapse of 4 days, these cells autolyze faster than the normal crypt epithelium. These curves correspond remarkably well, indicating that the increase in autolysis is due to a change in the cell protoplasm which persists

at least for 4 days after radiation. In all of the animals exposed to radiation, the crypt epithelium of the small intestine is completely autolyzed in 12 hours in the duodenum, ileum, and jejunum, while in the experiments with normal animals, the crypt epithelium is rarely autolyzed completely in 24 hours and usually the autolysis is only well advanced by that time.

Colon shows an increase in the speed of autolysis of the crypt epithelium and possibly of the tip epithelium, though the latter is less pronounced. The effect of the radiation seems to decrease or wear off slightly, for the speed of autolysis is somewhat slower and approaches the normal in the 3rd and 4th days after exposure to radiation. This may be connected with the fact that there is also relatively little injury to the colon, anatomically or histologically. The most pronounced increase in the speed of autolysis in the colon epithelium occurs, therefore, within the first 48 hours.

Spleen shows an apparent increase in the speed of autolysis, especially in the first 24 hours after exposure to the x-rays. The 72 hour curve is within normal limits, while both the 48 and the 96 hour curves show an increased speed in autolysis.

Pancreas shows a slight but definite increase in the speed of autolysis, especially during the first 48 hours after exposure. The 96 hour curves seem to indicate also that the speed of autolysis is increased, but the 72 hour curves are irregular as in the spleen series. The islands of Langerhans autolyze as a rule somewhat faster than the pancreatic parenchyma.

Kidney does not show changes in the speed of autolysis after exposure to radiation. The convoluted tubules are totally autolyzed in 8 hours. The other tubules and the glomeruli, while autolyzing more slowly, apparently digest at the normal rate. This would suggest that radiation has little effect upon the kidney.

Liver parenchyma shows a definite increase in the speed of autolysis, with a maximum in the specimens removed 2 hours after the radiation. The 24 and 48 hour experiments show less increase above normal.

DISCUSSION.

Many observers have studied ferment changes *in vitro* in normal organs or tumor tissues exposed to x-rays before the unit period of autolysis. Much of this work has been reviewed by Hall and Whipple and need not be discussed. Our experiments indicate that the spleen, liver, and pancreas taken from a radiated animal will show a more rapid autolysis than similar tissues from control normal animals under similar conditions of autolysis. The kidney shows no such change by the methods used. We may recall that the spleen shows obvious histological injury after exposure to the x-rays, but such changes are lacking in the liver, pancreas, and kidney.

The stomach and colon are much more resistant than the small intestine, but we shall publish experiments to show that large and permanent gastric and colon ulcers may be produced by the x-rays.

The *duration of the injury* done the body cells is of much interest to us. We note that abnormal autolysis of the spleen, liver, and pancreas is most striking within 24 hours after the radiation. After 48 hours, there is a distinct decrease in this reaction as observed in dogs killed 72 and 96 hours following the lethal dose of x-rays. In the small intestine, it is easy to demonstrate the abnormal autolysis in dogs even 96 hours after the x-ray exposure. This indicates in a sense that these cells are suffering from a persistence of the injury exerted by the x-rays, even during the 4th day after the exposure. It is significant that the clinical symptoms always reach a crisis on the 4th day. If this period is survived, the dog usually goes on to recovery.

We may note in passing that the cell necrosis and injury in the intestine as in the skin is associated with a minimal wandering cell reaction. This point deserves further study. We shall record also the fact that ulcers caused by the x-rays are as stubborn in healing in the stomach and intestine as is so well recognized in the skin.

SUMMARY.

Exposure to large doses of x-rays will cause notable increase in the speed of autolysis of the crypt or secretory epithelium of the dog's small intestine. These changes can be demonstrated readily in

material obtained from dogs killed 2, 24, 48, 72, or 96 hours after the initial radiation (Text-figs. 1 and 2).

In the radiated dogs the secretory crypt epithelium of the small intestine autolyzes first and the epithelium of the villi last, while the reverse is true in the normal control small intestine. These abnormalities of autolysis associated with lethal Roentgen ray exposures can be demonstrated for the small intestine over the whole 4 day period subsequent to radiation.

The colon shows little change and the stomach no demonstrable changes in autolysis under like conditions. The kidney likewise is negative.

The spleen, lymph glands, liver, and pancreas show a moderate increase in speed of autolysis in tissues taken from radiated animals within 48 hours of the initial exposure.

What the significance of this disturbance of cell ferments in the intestinal mucosa may be, we cannot pretend to say. At least these observations strengthen one's confidence in the profound functional disturbance of this important intestinal epithelium—a disturbance which we believe is responsible for the clinical abnormalities and fatal intoxication.

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A STUDY OF RENAL FUNCTION IN ROENTGEN RAY INTOXICATION.

RESISTANCE OF RENAL EPITHELIUM TO DIRECT RADIATION.

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In an earlier publication (Hall and Whipple (4)), the noticeable clinical similarity between intestinal obstruction and systemic x-ray intoxication has been pointed out. We have recorded elsewhere (10, 11) an interesting transient disturbance of renal function in the intoxication due to intestinal obstruction and in certain proteose intoxications. This disturbance of renal function is considerable and plays an important part in certain of the clinical reactions (intestinal obstructions). We believe this is the first instance of a well established functional depression of renal secretory function totally unassociated with any abnormalities of renal structure. Because of this peculiar disturbance of renal function in certain proteose intoxications, we took up a study of renal function in systemic x-ray intoxication with the keenest interest. We may say at once that our experiments show a totally different picture, with the renal function uninvolved in the toxic disturbance of general x-ray intoxication. There is a minimal disturbance of renal function by the direct action of very large doses of the hard x-rays but no anatomical evidence of cell injury. We feel confident that the clinician may use the x-rays over the kidney areas with confidence that renal tissue is resistant to the hard Roentgen rays. Our experiments indicate that the kidney epithelium is much more resistant to x-ray injury than is the epithelium of the small intestine.

Inspection of the literature shows that work has been done to determine possible injury of the kidney by the x-rays. However, most of the reports deal with the morphological changes produced or consist of clinical observations made by use

of the older, less reliable means of determining the presence of functional disturbances. Warthin (18) noted pathological changes in the kidneys of several leucemia patients who had received x-ray treatment before death and reports finding definite changes in the tubular epithelium following prolonged percutaneous irradiation of the kidneys of white mice and rats. Rosenstern (13), Linser and Helber (9), Schleip and Hildebrand (15), von Jaksch (6), Heymann (5), Příbram and Rotky (12), and others likewise claim to have found evidence of an x-ray nephritis. It has been thought by many that the inability of injured kidneys to do their work efficiently must be considered as an essential factor in the constitutional x-ray reaction. While Edsall and Pemberton (3) considered the primary intoxication to be quite independent of any direct effect of the x-rays upon the kidneys, they suggest that the kidneys may become overtaxed by the increased work thrown upon them to such an extent that their failure may secondarily intensify the symptoms of intoxication.

There are many workers, however, who have failed to find any definite clinical or postmortem evidence of an x-ray nephritis. Among those who have reported negative findings may be mentioned Krause and Ziegler (8) and Buschke and Schmidt (2). The latter workers incised the abdominal wall and exposed the kidneys of a number of rats directly to large doses of the x-rays. The animals died a few weeks later, two of them only showing albumin in the urine and pathological changes in the structure of the kidneys at autopsy. Since in these two cases the changes might well have been due to some complication following the operations, these workers concluded that the renal epithelium is very resistant to the x-rays and that consequently there is no such thing as an x-ray nephritis. Warthin, whose work is more recent and freer from complications, admits that the anatomical changes were so slight in his experimental animals that they might be overlooked unless special histological technique is employed. Krause (7) in 300 cases finds no albumin or leucocytes in the urine after radiation. Stephan (16) in a small series of cases claims to cure anuria and produce a diuresis in 24 hours by means of $\frac{1}{8}$ to $\frac{1}{4}$ of an erythema dose over the kidneys. This brief reference to the work previously done on the subject suffices to show the decided lack of agreement among those who have given any attention to it. There is obvious need for a thorough functional study of the problem with employment of the comparatively accurate methods now available for measuring the functional activity of the kidneys under conditions of carefully controlled x-ray administration.

Method.

Normal adult dogs were used exclusively in all of the experiments. The animals were selected after being under observation for a period of several days during which time the functional tests to be used in the subsequent experiments were performed on them. The data

recorded in this preliminary examination on each dog serve as a standard with which to compare results obtained in subsequent experiments. At the end of this fore period the animal was exposed to a carefully measured dose of x-rays, following which the renal function was determined daily for a period of a week or more.

The hard Roentgen rays of the Coolidge tube were used exclusively, the softer rays being filtered out with 2 mm. aluminum filters. In a very few cases only did we have leakage with slight tendency to pigmentation and denudation of the skin. All exposures were percutaneous and the distance from the target (anticathode) to the surface of the animal's body measured in all cases 25 cm. The ammeter was made to read at approximately 8 milliamperes in most of the experiments, although currents as high as 10 milliamperes and as low as 5 milliamperes were employed in a few instances. Our work confirms the observation of Hall and Whipple (4) that in dogs the toxicity of the hard rays increases with the increase in spark-gap. Our experience has shown that a 9 inch spark-gap is a convenient one for producing the desired experimental intoxication and that this required an E. M. F. of about 90 kilovolts. With a few exceptions, this voltage was employed.

In a part of the experiments moderate sized sublethal doses of x-rays were given at intervals over a considerable period of time, a short rest on a mixed diet being given between exposures. In the remainder, huge and frequently fatal doses were given singly or in succession. It was considered that between these extremes no impairment of function, whether prompt or delayed in its appearance, could escape detection. In order to understand the nature or the mechanism of any disturbance which might be observed, different regions of the body were exposed separately in the various experiments.

For a period of 1 to 3 days preceding and 5 to 6 days subsequent to the radiation, the dogs were given only water. During resting intervals, the dogs received the usual kennel mixed diet of table scraps, cooked meat, bread, rice, bones, and so forth.

The methods chosen for the measurement of the renal function were those employed by us in studies on the renal function in intestinal obstruction (10); namely, the phenolsulfonephthalein elimination test (14) and that of determining the capacity of the kidneys to ex-

crete urea. The former method was carried out according to the procedure outlined by its authors, the Duboscq colorimeter being used for the determination of the dye. For the latter we made a daily determination of the blood urea in all experiments and determined in many cases the ratio

$$\frac{\text{Urea in 1 hr.'s urine}}{\text{Urea per 100 cc. of blood}}$$

after the intravenous injection of comparatively large amounts of urea in saline solution. A decrease in this ratio indicates a definite impairment of kidney function as shown by Addis and Watanabe (1). The tests were made after the exposure to the x-rays exactly as in the fore period except that in cases of extreme intoxication with resulting loss of fluid from the body, more fluid was given intravenously in order that the loss might be made up as far as possible. The urea of both the blood and the urine was determined by the Van Slyke-Cullen modification of the Marshall urease method (17). Morphine sulfate ($\frac{1}{8}$ to $\frac{1}{4}$ gr. doses) was administered subcutaneously 1 hour before the animal was placed upon the x-ray table to insure quiet during the period of exposure.

EXPERIMENTAL DATA.

The experimental data assembled below consist of three types of experiments. The first includes a group of observations on several different animals in which *sublethal doses of x-rays* were given at intervals over relatively long periods of time. In these experiments, the thorax and the abdomen, including the kidneys, were alternately exposed. The purpose of this series was to ascertain whether or not repeated x-ray treatment over a long period of time is capable of producing any gradual changes in the renal function. Many have feared the possibility of cumulative action resulting in permanent damage to the kidneys.

Dog 19-85 (Tables I and II). Autopsy Record.—Dog sacrificed under ether anesthesia 4 days after the last radiation, Apr. 5. The kidneys in gross are quite normal. Bladder normal. Histological sections show normal stroma, vessels, and glomeruli. There are no scars. Convoluted tubules are normal.

TABLE I.

*Large Sublethal Doses of X-Rays Given over Abdomen and Thorax.
Renal Function Unimpaired.*

Dog 19-85. Adult female spitz.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	<i>lbs.</i>	<i>mg.</i>	<i>per cent</i>	
Nov. 30	24.9	23	75	Normal.
Nov. 30	190 milliamperere minutes, x-rays over abdomen and kidneys; 90 kilovolts; 10 milliamperes.			
Déc. 1	24.7	22	71	Normal.
" 3	24.2	36	74	"
" 5	23.7	34	54	Slight diarrhea.
" 6	23.2	36	55	No diarrhea. Normal.
" 7	22.7	40	63	Normal.
Dec. 16	220 milliamperere minutes, x-rays over thorax and neck; 90 kilovolts; 10 milliamperes.			
Dec. 17	24.3	32	62	Normal.
" 18	23.9	30	69	—
" 19	23.1	42	57	—
" 20	23.0	31	52	—
" 21	22.9	30	67	Normal.
Dec. 31	350 milliamperere minutes, x-rays over abdomen and back; 90 kilovolts; 8 milliamperes.			
Jan. 1	22.6	—	67	Normal.
" 2	22.0	22	67	—
" 3	21.8	20	71	—
" 4	21.2	23	63	Normal.
" 5	20.8	30	67	—
" 6	20.4	28	63	—
Feb. 4	300 milliamperere minutes, x-rays over abdomen and back; 95 kilovolts; 8 milliamperes.			
Feb. 8	23.0	34	63	Normal.
" 9	23.0	30	63	—
" 10	22.6	28	66	—
" 11	22.4	24	64	—
" 12	22.2	25	63	—

Mixed diet in intervals between experiments; no food for 5 days following exposures.

It will be seen from Tables I to IV that the experiments of this group are a unit in showing that the excretory function of the kidneys, as measured by their ability to eliminate a foreign substance such as phenolsulfonephthalein or the normal urinary constituent, urea, is not disturbed by this procedure. It is evident that with the doses given there is no sign of any cumulative effect being produced, whether the thorax or abdomen or both be exposed.

TABLE II.
*Large Sublethal Doses of X-Rays Given over Abdomen and Thorax.
Renal Function Unimpaired.*

Dog 19-85. Adult female spitz.

Date.	Hr.	Urea in- jected.	Urea ex- creted per hr.	Blood urea per 100 cc.	Ratio.	Phtha- lein elim- ination in 2 hrs.	Remarks.
		gm.	gm.	mg.		per cent	
Dec. 21	9.00-10.00	—	—	30	—	—	Normal.
	10.00-12.00	20	3.59	262	13.7	67	—
Mar. 4	350 milliamperere minutes, x-rays over thorax; 95 kilovolts; 5 milliamperes.						
Mar. 7	9.00-11.00	—	—	28	—	63	Normal.
" 8	—	—	—	27	—	64	—
" 9	10.00-12.00	20	3.78	270	14.0	67	Normal.
Apr. 1	350 milliamperere minutes, x-rays over abdomen; 105 kilovolts; 5 milliamperes.						
Apr. 4	12.00-2.00	—	—	39	—	65	Moderate intoxication.
" 5	3.00-5.00	20	3.42	278	12.3	—	Same condition.

The second type of experiment is represented by the observations made on Dogs 19-127 and 19-140 (Tables V to VIII) in which a lethal dose of the x-rays was given over the abdomen with the kidneys and the remainder of the body protected from the direct rays by the leaden sheets. This type of experiment was designed to demonstrate whether or not a poisonous substance capable of injuring the kidneys is produced in the irradiated gastrointestinal tract and carried by the blood stream to the kidneys. Reference to the tables shows that the function of the kidneys remains entirely normal after the exposure,

TABLE III.

Repeated Sublethal Doses of X-Rays over Thorax and Abdomen.

Renal Function Unchanged.

Dog 19-78. Adult female Airedale. (Autopsy, see Table XV.)

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	<i>lbs.</i>	<i>mg.</i>	<i>per cent</i>	
Nov. 23	28.5	30	68	Normal.
Nov. 25	190 milliampere minutes, x-rays over thorax and neck; 85 kilovolts; 5 milliamperes.			
Nov. 26	28.1	18	63	Normal.
" 27	27.5	22	57	—
" 28	27.4	20	60	—
" 29	27.1	—	58	—
" 30	26.5	33	58	—
Dec. 1	26.4	28	60	—
" 2	26.4	23	68	No food since exposure.
" 5	26.0	20	60	Mixed diet.
" 10	27.5	—	62	—
Dec. 10	190 milliampere minutes, x-rays over abdomen and back; 90 kilovolts; 5 milliamperes.			
Dec. 11	27.5	40	59	Normal.
" 12	27.0	40	59	—
" 13	26.2	40	54	—
" 14	26.0	41	59	—
" 15	25.7	36	54	—
" 16	24.9	22	59	—
Dec. 27	240 milliampere minutes, x-rays over thorax; 90 kilovolts; 5 milliamperes.			
Dec. 28	26.6	36	64	Normal.
" 29	26.5	47	64	—
" 30	26.3	50	62	—
" 31	25.5	43	63	—
Jan. 1	25.0	33	59	—

Dog not fed for day or two before exposure and for 5 days after each radiation.

although the dose of x-rays produced profound intoxication and death. The evidence against any indirect action of the x-rays upon the kidney function or upon the structure of the kidney is very clear-cut. The blood urea showed a definite increase in both cases, but this was due

to accelerated tissue destruction rather than to retention, as is shown by the fact that the urea ratio (Tables VI and VIII) remained normal

TABLE IV.
*Repeated Increasing Doses of X-Rays over Thorax and Abdomen.
Renal Function Undisturbed.*

Dog 19-50. Young adult female. (Autopsy, see Table XVI.)

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimina- tion in 2 hrs.	Remarks.
	<i>lbs.</i>	<i>mg.</i>	<i>per cent</i>	
Dec. 5	30.40	28	74	—
Dec. 5	210 milliampere minutes, x-rays over thorax; 90 kilovolts; 10 milli- amperes.			
Dec. 6	29.50	22	72	Normal.
" 7	29.30	31	66	—
" 8	28.20	31	63	—
" 9	27.00	34	55	—
" 10	26.40	34	63	—
" 12	26.50	—	—	—
" 14	26.50	28	70	—
" 15	27.10	33	—	—
Dec. 16	210 milliampere minutes, x-rays over abdomen and back; 90 kilo- volts; 10 milliamperes.			
Dec. 17	27.15	37	56	Normal.
" 18	27.00	31	68	—
" 19	26.70	34	65	—
" 20	25.40	30	63	—
Dec. 31	350 milliampere minutes, x-rays over thorax; 90 kilovolts; 7.5 milli- amperes.			
Jan. 1	26.70	—	72	Normal.
" 2	26.50	28	67	—
" 3	26.00	26	61	—
" 4	25.50	22	61	—
" 5	25.40	24	63	—
" 6	25.30	20	66	—
" 7	25.00	21	63	—

even when additional urea was injected intravenously. The results indicate the absence of any hypothetical nephrotoxic substance in the blood during the period of intoxication, and thus point out a

TABLE V.

*Lethal Dose of X-Rays over Abdomen with Kidneys Shielded.
Renal Function Unchanged.*

Dog 19-127. Adult male mongrel.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	<i>lbs.</i>	<i>mg.</i>	<i>per cent</i>	
June 9	28.4	32	74	Normal.
“ 10	28.3	30	72	
June 10	504 milliampere minutes, x-rays over abdomen, kidneys completely shielded; 90 kilovolts; 8 milliamperes.			
June 11	27.8	27	76	Normal.
“ 12	26.3	35	67	Quiet.
“ 13	26.1	44	68	Dull and weak.
“ 14	25.5	46	66	Intoxication increasing.
“ 15	—	53	59	Grave intoxication and col- lapse. Death in night.

TABLE VI.

*Lethal Dose of X-Rays over Abdomen with Kidneys Shielded.
Renal Function Unchanged.*

Dog 19-127. Adult male mongrel.

Date.	Hr.	Urea in- jected.	Urea ex- creted per hr.	Blood urea per 100 cc.	Ratio.	Phtha- lein elim- ination in 2 hrs.	Remarks.
		<i>gm.</i>	<i>gm.</i>	<i>mg.</i>		<i>per cent</i>	
June 9	8.50- 9.50	—	0.160	32	5.0	—	—
	10.00-11.00	20	2.382	240	9.9	74	Normal.
	11.00-12.00	—	1.856	201	9.2	—	—
June 10	504 milliamperere minutes, x-rays over abdomen, kidneys shielded; 90 kilovolts; 8 milliamperes.						
June 14	8.45- 9.45	—	0.144	46	3.1	—	—
	10.00-11.00	20	2.215	237	9.3	66	Severe intoxication.
	11.00-12.00	—	1.693	192	8.8	—	—

TABLE VII.

*Lethal Dose of X-Rays over Abdomen with Kidneys Shielded.
Renal Function Unchanged.*

Dog 19-140. Young adult female collie.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	<i>lbs.</i>	<i>mg.</i>	<i>per cent</i>	
July 7	21.7	34	70	—
" 8	20.5	32	72	Normal.
July 8	450 milliamperere minutes, x-rays over abdomen, kidneys shielded; 90 kilovolts; 8 milliamperes.			
July 9	19.7	30	70	Normal.
" 10	19.3	34	63	Dull and weak.
" 11	19.0	43	63	Severe intoxication; bloody diarrhea; death in night.

TABLE VIII.

*Lethal Dose of X-Rays over Abdomen with Kidneys Shielded.
Renal Function Unchanged.*

Dog 19-140. Young adult female collie.

Date.	Hr.	Urea in- jected.	Urea ex- creted per hr.	Blood urea per 100 cc.	Ratio.	Phtha- lein eli- mi- nation in 2 hrs.	Remarks.
		<i>gm.</i>	<i>gm.</i>	<i>mg.</i>		<i>per cent</i>	
July 8	7.55- 8.55	—	0.160	32	5.0	—	—
	9.00-10.00	20	3.028	244	12.4	72	Normal.
	10.00-11.00	—	2.353	219	10.7	—	—
July 8	450 milliamperere minutes, x-rays over abdomen, kidneys shielded; 90 kilovolts; 8 milliamperes.						
July 11	8.30- 9.30	—	0.206	43	4.8	—	—
	10.00-11.00	20	2.744	240	11.4	68	Typical x-ray intoxica- tion.
	11.00-12.00	—	2.366	213	11.1	—	—

difference between this type of intoxication and that of acute intestinal obstruction in which a transient impairment of kidney function is constantly found.

TABLE IX.

*Large Doses of X-Rays Directly over Kidneys with Abdomen Shielded.
Kidney Function Slightly Impaired.*

Dog 19-117. Adult female setter.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	<i>lbs.</i>	<i>mg.</i>	<i>per cent</i>	
July 28	38.8	32	67	Normal.
July 28	456 milliampere minutes, x-rays directly over kidneys, other parts shielded; 90 kilovolts; 8 milliamperes.			
July 29	38.8	30	68	Normal.
" 30	38.4	29	66	—
" 31	38.0	27	66	Bread and milk diet.
Aug. 1	37.6	28	63	Dog a little sick.
" 2	37.4	24	63	—
" 3	37.8	—	—	—
" 4	37.4	26	48	—
" 5	37.0	33	32	—
" 6	36.4	—	39	—
" 10	—	33	49	Normal.
" 11	36.5	33	56	—
Aug. 12	512 milliampere minutes, x-rays over both kidneys with other parts shielded; 90 kilovolts; 8 milliamperes.			
Aug. 13	36.3	26	59	—
" 14	36.2	30	—	Dog fed; good condition.
" 15	36.0	30	55	—
" 16	36.1	—	56	—
" 17	36.2	32	48	—
" 18	36.0	38	45	Small amount of vomitus.
" 19	36.0	—	42	Moderate diarrhea.
" 20	35.2	32	58	Clinical improvement.
" 21	35.0	—	—	Normal.

Dog 19-127 (Tables V and VI). Autopsy Record.—Dog died in night of 4th day after exposure to a lethal dose of x-rays given over abdomen with shielding of the kidneys. Autopsy next morning. Kidneys and bladder normal in gross. Histological sections show some postmortem changes in the epithelium of the

convoluted tubules. The stroma and glomeruli are normal. There are no scars. The vessels are filled with blood.

The third type of experiment is represented by the series of observations on Dogs 19-117, 19-129, and 18-115 (Tables IX to XIV).

TABLE X.
*Large Dose of X-Rays Directly over Kidneys with Abdomen Shielded.
Kidney Function Slightly Impaired.*

Dog 19-117. Adult female setter.

Date.	Hr.	Urea in- jected.	Urea ex- creted per hr.	Blood urea per 100 cc.	Ratio.	Phtha- lein elim- ination in 2 hrs.	Remarks.
		gm.	gm.	mg.		per cent	
July 14	12.50-1.50	—	0.170	30	5.6	—	—
	2.00-3.00	20	1.799	158	11.4	74	Normal.
	3.00-4.00	—	1.323	142	9.3	—	—
July 15	456 milliamperere minutes, x-rays over thorax and neck; 90 kilovolts; 8 mil- liamperes.						
July 18	8.45- 9.45	—	0.160	27	5.9	—	—
	10.00-11.00	20	1.826	159	11.5	67	Normal.
	11.00-12.00	—	1.357	144	9.4	—	—
July 28	456 milliamperere minutes, x-rays over kidneys, other parts shielded; 90 kilovolts; 8 milliamperes.						
Aug. 11	2.50-3.50	—	0.160	33	4.8	—	—
	4.00-5.00	20	1.505	173	8.7	56	Not clinically sick.
	5.00-6.00	—	1.182	163	7.3	—	—
Aug. 12	512 milliamperere minutes, x-rays over kidneys, other parts shielded; 90 kilovolts; 8 milliamperes.						
Aug. 18	1.45-2.45	—	0.174	38	4.5	—	—
	3.00-4.00	20	1.342	179	7.4	45	Dull. Small amount vomitus.
	4.00-5.00	—	1.316	167	7.8	—	—

In these the kidneys were exposed to huge doses of the x-rays directly while the remainder of the abdomen and the thorax were completely shielded. Large doses were repeatedly given in the case of the first animal while large single doses only were given in the other cases.

TABLE XI.

*Large Dose of X-Rays over Kidneys with Abdomen Shielded.
Kidney Function Slightly Impaired.*

Dog 19-129. Adult male mongrel.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	<i>lbs.</i>	<i>mg.</i>	<i>per cent</i>	
Aug. 6	67.9	29	66	Normal.
" 7	67.4	28	67	—
Aug. 8	512 milliamperes minutes, x-rays over thorax; 90 kilovolts; 8 milliamperes.			
Aug. 10	66.7	26	66	Normal.
" 12	65.0	29	63	—
" 14	64.8	25	64	Mixed diet.
Aug. 19	512 milliamperes minutes, x-rays directly over kidneys, with other parts shielded; 90 kilovolts; 8 milliamperes.			
Aug. 19	63.0	29	67	Normal.
" 22	61.7	38	53	Slightly dull.
" 24	59.7	46	44	Little vomitus.
" 26	59.3	44	54	Slightly dull.
" 28	59.0	32	58	Normal.

TABLE XII.

*Large Dose of X-Rays over Kidneys with Abdomen Shielded.
Kidney Function Slightly Impaired.*

Dog 19-129. Adult male mongrel.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		<i>gm.</i>	<i>gm.</i>	<i>mg.</i>		<i>per cent</i>	
Aug. 19	8.40- 9.40	—	0.142	29	4.9	—	—
	10.00-11.00	20	1.368	143	9.5	66	Control.
	11.00-12.00	—	1.083	129	8.4	—	—
Aug. 19	512 milliamperes minutes, x-rays over kidneys with abdomen shielded; 90 kilovolts; 8 milliamperes.						
Aug. 26	—	—	—	47	—	—	—
	1.00-2.00	20	1.124	161	6.9	47	Slightly dull.
	2.00-3.00	—	0.933	150	6.2	—	—

Dog 19-129 showed no signs of intoxication, whereas No. 19-117 showed a slight reaction and No. 18-115 was markedly intoxicated. It is probable that in the case of No. 18-115 a small portion of the intestine was included in the area of exposure. It is to be noted that the doses given over the kidneys in this group of experiments were at least 25 per cent greater than the lethal dose over the abdomen.

TABLE XIII.
*Large Dose of X-Rays over Kidneys with Abdomen Shielded.
Kidney Function Slightly Impaired.*

Dog 18-115. Adult female bulldog. *				
Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	mg.	per cent	
Aug. 23	21.5	30	68	Normal.
" 24	20.2	27	66	"
Aug. 27	512 milliampere minutes, x-rays over kidneys with remainder of body shielded; 90 kilovolts; 8 milliamperes.			
Aug. 28	19.4	32	62	Normal.
" 29	19.2	31	54	—
" 30	19.0	33	58	Normal.
" 31	—	—	58	Slightly dull.
Sept. 1	18.3	52	40	Quiet; vomitus and tarry stools.
" 2	17.9	43	—	Tarry stools and vomitus.
Sept. 3	300 milliampere minutes, x-rays over abdomen alone; 90 kilovolts; 8 milli-amperes.			
Sept. 4	17.2	36	53	Vomits food; tarry stools.
" 5	16.7	44	46	Weak and dull.
" 8	—	—	—	Found dead in cage.

Dog 19-117 (Tables IX and X). *Autopsy Record.*—Dog sacrificed under ether Aug. 24 and autopsy done at once. Kidneys and bladder normal in gross. Histological sections show normal glomeruli, vessels, and stroma. There are no scars. Convoluted tubules show normal epithelium. There are a few pale hyaline casts in the collecting tubules of the pyramids.

Dog 19-129 (Tables XI and XII). *Autopsy Record.*—Dog sacrificed with ether anesthesia Aug. 29 and autopsy done at once. Kidneys show definite chronic nephritis with a large cyst in the left kidney. There is a chronic granular cystitis. Histological sections show a marked cellular pyelitis in both kidneys. There are

a few cellular scars in the cortex and some large fibrous scars (arteriosclerotic) involving a considerable area of the cortex and showing tubular and glomerular destruction. There is no evidence of acute nephritis. Outside the scar areas, the glomeruli and tubules appear to be quite normal. This picture is often found in the kidneys of old dogs and cannot in any way be related to the x-ray exposures. This abnormal kidney seems to be no more susceptible to radiation than the normal kidney.

Dog 18-115 (Tables XIII and XIV). Autopsy Record.—Dog found dead in cage Sept. 8 and autopsy done at once. Kidneys are normal in gross. Histological sections show normal glomeruli and vessels. There are a few cellular scars in cortex. The convoluted tubules show a slight postmortem change, but otherwise the tubules are normal.

TABLE XIV.

*Large Dose of X-Rays over Kidneys with Abdomen Shielded.
Kidney Function Slightly Impaired.*

Dog 18-115. Adult female bulldog.

Date.	Hr.	Urea in- jected.	Urea ex- creted per hr.	Blood urea per 100 cc.	Ratio.	Phtha- lein elim- ination in 2 hrs.	Remarks.
		gm.	gm.	mg.		percent	
Aug. 24	2.30-3.30	—	0.153	27	5.6	—	—
	4.00-5.00	20	2.562	197	13.1	66	Control.
	5.00-6.00	—	1.998	181	11.0	—	—
Aug. 27	512 milliamperere minutes, x-rays over kidneys with remainder of body shielded; 90 kilovolts; 8 milliamperes.						
Sept. 1	1.30-2.30	—	0.203	52	3.9	—	—
	3.00-4.00	20	2.014	217	9.2	40	Dog is weak. Tarry stools and vomitus.
	4.00-5.00	—	1.683	179	9.4	—	—

Tables IX to XIV inclusive, in which the data of these experiments are recorded, show that the direct exposure of the kidneys to massive doses of the x-rays gives rise to a moderate but definite depression of the kidney function. In Tables XV and XVI are shown the results of two experiments differing slightly from the foregoing in that the kidneys and the intestines were together exposed to large doses of x-rays, the thorax only being protected. A definite decrease in the 2 hour output of phenolsulfonephthalein and a corresponding drop in the urea ratio indicate a moderate impairment of renal function.

TABLE XV.

*Lethal Dose of X-Rays over Kidneys and Abdomen.
Renal Function Moderately Impaired.*

Dog 19-78. Adult female Airedale.

Date.	Hr.	Urea in-jected.	Urea ex-creted per hr.	Blood urea per 100 cc.	Ratio.	Phtha-lein elimi-nation in 2 hrs.	Remarks.
		gm.	gm.	mg.		percent	
Dec. 7	1st	—	—	24	—	—	—
	2nd	20	1.826	152	12.0	69	Control.
	3rd	—	1.594	131	12.1	—	—
Mar. 18	350 milliampere minutes, x-rays over abdomen and kidneys; 95 kilovolts; 8 milliamperes.						
Mar. 20	—	—	—	32	—	55	Slightly dull.
" 21	—	—	—	45	—	40	Intoxication progressing.
Mar. 22	1st	—	—	69	—	—	—
	2nd	20	1.861	258	7.2	38	Marked intoxication; bloody diarrhea.
	3rd	—	1.443	239	6.0	—	—

Dog killed under ether at end of observation.

TABLE XVI.

*Lethal Dose of X-Rays over Kidneys and Abdomen.
Renal Function Slightly Impaired.*

Dog 19-50. Young adult female.

Date.	Hr.	Urea in-jected.	Urea ex-creted per hr.	Blood urea per 100 cc.	Ratio.	Phtha-lein elimi-nation in 2 hrs.	Remarks.
		gm.	gm.	mg.		percent	
Feb. 10	1st	—	0.240	29	4.8	—	—
	2nd	20	1.983	180	11.0	66	Control.
	3rd	—	1.529	156	9.8	—	—
Feb. 11	340 milliampere minutes, over abdomen and kidneys; 95 kilovolts; 8 milli-amperes.						
Feb. 13	—	—	—	42	—	56	Dull; vomitus and diarrhea.
Feb. 14	1st	—	0.328	78	4.2	—	—
	2nd	20	2.146	280	7.66	40	Frequent diarrhea and vomiting.
	3rd	—	1.543	249	6.19	—	—

From the results of the experiments outlined above we assume that the direct action of the x-rays upon the kidneys is responsible for this depression of activity.

Dog 19-78 (Tables III and XV). Autopsy Record.—Dog killed under ether at end of experiment given in Table XV. Autopsy done at once but only the renal findings given here. Kidneys are normal in gross. Histological sections show normal glomeruli, vessels, and stroma. There are no scars. Convolutated tubules are normal. The epithelium of the loops of Henle shows a frothy looking protoplasm but this picture is seen in control dogs.

Dog 19-50 (Tables IV and XVI). Autopsy Record.—Dog killed under ether anesthesia at end of last experiment (Table XVI) and autopsy at once. Only the renal findings are given here. Kidneys and bladder normal in gross. Histological sections show normal glomeruli and vessels. There are a few small cellular scars in the cortex. Tubules are normal.

Reference to the autopsy findings in the various dogs used in the experiments shows that no constant anatomical change is seen in the kidneys which would serve to distinguish them from a series of normal control kidneys chosen at random. We did not use the refined histological technique recommended by Warthin, but we are inclined to believe that much of the histological evidence submitted in favor of x-ray injury of the kidney is due to kidney abnormalities present *before the x-ray exposures*. Such abnormalities would be revealed by a careful study of suitable controls.

SUMMARY.

Our experiments give no support to the current belief that an x-ray nephritis may be produced by direct or indirect action of the hard Roentgen rays.

Moderate doses of x-rays given *repeatedly* over considerable periods of time have no demonstrable influence on renal function or renal structure.

With x-ray exposures of the abdomen and shielding of the kidneys fatal intoxication may be produced without the slightest disturbance of kidney function as measured by the ability of the kidney to eliminate phenolphthalein and urea.

Large doses of the x-rays given directly over the kidney may cause a slight but distinct lowering of renal function which lasts for a

period of a few days. We have been unable to recognize any corresponding histological change.

We feel that the usual therapeutic doses of the x-rays can be given over the kidneys without apprehension. The renal tissue is much more resistant to x-rays than is the epithelium of the small intestine.

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THE RELATION OF THE HYPOPHYSIS TO ANTIBODY PRODUCTION.

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The important part that many of the endocrine organs play in development and function suggests the possibility that they may also enter into the mechanism of the body which develops resistance to infection. Up to the present time there is little positive evidence that such is the case. It was felt, however, that certain of the more inaccessible glands of internal secretion, whose function in relation to immunity had not been studied, might yield valuable information, and this study of the pituitary gland was made as part of a general investigation into this field (6).

The experimental work so far has been chiefly of a physiological nature concerning the part the pituitary plays in growth, metamorphosis, sugar tolerance, and sexual activity, and its relation to acromegaly, dyspituitarism, and diabetes mellitus. The functions of the two parts of the gland have been elaborately defined (13, 16). More recently the interrelationship of this gland to the other ductless glands has been much discussed. That total hypophysectomy is incompatible with life¹ would seem to be proved by the work of Cushing and his collaborators (3, 4) and Paulesco (17). The striking physical changes in growth, development, nutrition (3), and general metabolism (2) with partial hypophysectomy (1) and by feeding pituitary gland extract (9, 10, 20) are now accepted. We have not been able to find, however, any direct, quantitative, experimental studies of the relation of the hypophysis to infectious diseases. Aschner (1) thought his partially hypophysectomized dogs succumbed more easily to disease than normal dogs, and certainly many contracted pneumonia. Cushing's experiments (3) seem to bear this out. There are, however, no clinical observations that would seem to indicate a lessened or increased resistance to infectious disease

¹ The work of Gemelli (8), Aschner (1), and Handelsmann and Horsley (10) disputes this, but their extirpation methods were so injurious and uncontrolled as to appear unreliable. Our own studies on the guinea pig agree with those of Paulesco and Cushing.

with pituitary disorders.² Gay and Rusk (7) in a study of the locus of antibody production draw no definite conclusion, but the evidence would seem to indicate that the spleen and the lymphatic system play the most important part. The work of Murphy and his associates (15), Hektoen (11), Hektoen and Curtis (12), and Morris and Bullock (14) appears to corroborate this view. The experiments of Topley (19) appear to disprove the contention that antibodies are formed at the point of inoculation.

Problem and Methods.

Guinea pigs were selected as the experimental animal because of their already standardized reactions in immunological studies. An operative technique was elaborated for partial hypophysectomy. In such animals the serum reactions of typhoid agglutination, hemagglutination, and hemolysis were studied. These studies were completed in the following series.

Series 1. The Production of Antibodies after Hypophysectomy.—Partial hypophysectomized, control operation animals and normal controls were immunized to *Bacillus typhosus*. Subsequent titers indicated the degree of immunity produced.

Series 2. The Effect of Hypophysectomy on Antibody Production.—This was measured by the change in height of the antibody curve. Guinea pigs were immunized to (1) *Bacillus typhosus* and (2) hen red blood corpuscles, and then submitted to partial hypophysectomy. Normal and control operation animals were used. Titration of agglutinins and hemolysins was carried out at intervals before and after operation.

Series 3. The Effect of Feeding and Injecting Pituitary Extract on the Production of Antibodies.—Animals were immunized, then fed continuously pituitary extract or submitted to repeated injection of the extract, and their subsequent titers determined.

Operative Technique.

Adult guinea pigs weighing from 300 to 400 gm. were used. In such animals the pituitary is relatively large, weighing about 0.025 gm. (Vanderburgh (21)). It lies in a shallow dural pocket and has the characteristic histologic appearance of other mammalian hypophyses. After some difficulties the following operative attack was chosen as giving an approach through which a definite portion of the

² Personal communication from Dr. Harvey Cushing.

gland could be removed and in which success was obtained in about 75 per cent of the animals used. The animal was placed on an electrically heated pad, kept at a fixed temperature, and fastened down to a small raised table. Anesthesia was induced by means of a fine rubber tube passed into the posterior pharynx through which a small pump forced ether vapor (5). Morphia 0.002 gm. and atropine 0.004 gm. were given 15 to 30 minutes before anesthesia. After shaving the head and cleaning the scalp with 1:1,000 bichloride a wet gauze covering was placed over the head and a sterile towel over the body of the animal. The gauze over the vertex of the skull was cut and an incision in the skin was made from just behind the eyes to the occipital protuberance and carried through to the bone. The tissue flaps on both sides were then dissected up and held away by the weight of a single hemostat. The parietal bone was perforated and all the bone over both hemispheres was removed as far anteriorly as the frontoparietal synostoses and posteriorly to the occipital bone. On the right side the decompression was carried to the temporoparietal juncture and on the left well into the temporal bone. The dura was left intact and the longitudinal sinus which lies in it, free from the bone, remained uninjured. The dura was then incised low down in the left temporal region, a small spatula introduced beneath the temporal lobe, and the brain elevated and slightly dislocated towards the right side. (The removal of bone on the right side was done to permit this.) A view was thus obtained of the pituitary fossa, the small pinkish body lying surrounded on both sides and posteriorly by large venous sinuses. The stalk enters at the anterior end of the gland, running slantwise to the posterior region. The dura over the middle of the hypophysis was then nicked with a small hook knife and with a small double spoon forceps fragments of the gland were removed. Unless the sinus was injured, which was fatal, there was little hemorrhage. Warm normal saline solution on cotton pledgets was used for sponging and hemostasis. After removal of the gland fragment, the brain was allowed to return to its position and the skin carefully sutured with two layers of fine interrupted silk sutures. This was covered by a cocoon dressing. The operation consumed between 30 and 45 minutes.³ Control animals in which the same operation, without removal of the pituitary, was performed, were prepared. The above procedure was carried out through the step in which the dura over the sella was cut and then closure completed, thus subjecting the control animals to approximately the same amount of trauma and anesthesia.

Total hypophysectomy proved fatal, the animals almost at once entering into a lethargic, partly comatose state, refusing food and water and dying in 24 to 48 hours. All fragments removed were

³ The chief complication in recovered animals was an ocular palsy, the result apparently of injuring the third cranial nerve which lies practically on the hypophysis but which we attempted to push to one side before the final step of enucleation.

studied histologically, and no animals are reported in which there was not this proof that at least one-half of the gland, which always consisted of fragments of both pars anterior and posterior, was removed. Autopsy controls were used to establish further the efficacy of this method of attack. The surviving animals within 24 hours made excellent recoveries, but lost 50 to 100 gm. in weight in the first few days. After this they regained weight and appeared, except for a rare ocular palsy, as active and vigorous as the normal controls. In many cases the gain in weight was rapid and even above that of the normal animals.

Technique of the Serum Reactions.

Typhoid Agglutinins.—The Sen strain of *B. typhosus* was used.⁴ It had been grown on artificial media for years but still combined a ready agglutinability with a high toxicity for guinea pigs. It was carried along throughout these experiments by repeated daily transplants on agar and bouillon with occasional plating to determine its purity. A vaccine was prepared by suspending the 24 hour growth in six Blake bottles in salt solution, killing the organisms by adding chloroform, drying them in a vacuum, and then grinding up the bacteria in a sterile mortar. Weighed quantities were then suspended in normal salt solution and tricresol was added for maintaining sterility. Experimentation with vaccine and living organisms determined that the greatest reaction was produced by the following inoculations given at 3 and 4 day intervals: first injection, 0.002 gm. of vaccine subcutaneously; second injection, 0.002 gm. intraperitoneally; third injection, $\frac{1}{8}$ of a 20 hour growth of living organisms on an agar slant intraperitoneally; and fourth injection, $\frac{1}{8}$ of a 20 hour growth of living organisms on an agar slant intraperitoneally. By the 10th day after the last injection agglutinin titers ranged from 1:1,000 to 1:6,000. In Text-figs. 1 and 2 the normal control curve represents the average of over twenty-five animals. In performing agglutination tests 1 drop of a salt suspension of a 24 hour growth of the bacteria on agar was added to 1 cc. of successive dilutions of serum. Serum was obtained by cardiac puncture, 4 cc. of blood being withdrawn, and the serum inactivated. Titration was recorded after 2 hours at 37°C., after 2 hours at room temperature, and after the tubes had been in the ice box over night. 4 cc. of blood proved sufficient for both agglutination and complement fixation tests. The latter tests, however, although completed with almost all animals used, are not reported since the concentration of complement fixation bodies was not sufficient to ensure reliable comparative results.

⁴ This strain was obtained from Dr. Carroll G. Bull.

Hemagglutinins and Hemolysins.—Another series of guinea pigs with normal controls, partially hypophysectomized controls, and operation controls was immunized to hen red blood corpuscles for the study of hemagglutinins and hemolysins. At 4 day intervals all animals were given intraperitoneally 1 cc. of washed hen corpuscles made up to the original blood volume. At the end of 1 week serum was obtained and the quantity of reaction determined. In testing for hemagglutinins 1 drop of a 10 per cent suspension of washed hen corpuscles was added to 1 cc. volumes of successively diluted, inactivated serum. Readings were made as with the typhoid studies. For hemolysins the usual technique of the Wassermann reaction was followed with 0.25 cc. volumes of inactivated serum, fresh guinea pig complement 1:10, a 5 per cent suspension of hen corpuscles, and 0.5 cc. of saline solution. The tubes were then incubated for 1½ hours, being shaken after 30 minutes and 1 hour. Readings were made after 1½ hours and after standing in the ice box over night. The usual controls were performed with each test.

The several series were performed with the above methods and complement deviation studies were also made. The latter, however, are not reported because of the low and variable nature of the titers resulting; it appears probable that for this test insufficient antibodies are manufactured. Operative difficulties now and then caused the loss of valuable animals but a sufficient number was always used in each experimental unit to ensure a satisfactory number of complete studies. As a rule, the experimental unit consisted of two normal animals, two partially hypophysectomized animals, and two operative control animals for Series 1. Series 2 experiments, however, were usually started with eight guinea pigs, since the operation was completed only when an immunity had been established and titers determined, and unless sufficient animals were provided the loss of a guinea pig at operation would vitiate that particular experimental unit. Before commencing with typical experimental units, the operative technique was perfected and a supply of hypophysectomized and control operation animals provided. The agglutinin, hemolysin, and hemagglutinin reactions were studied in normal guinea pigs and the quantitative reaction was well standardized. The immunity production in partially hypophysectomized and operative control animals showed no appreciable difference in these preliminary studies. In the actual experimental units guinea pigs as near of a size and kind as possible were chosen. Because of the reported interrelation of the

pituitary body with the sex glands (9) and the reported increased reaction in menstruating women to typhoid vaccine, preliminary studies were made which ruled out any appreciable influence that such sexual periods might have on antibody production. Neither menstruation⁵ nor the pregnant state appeared to have any influence on the antibody curve.

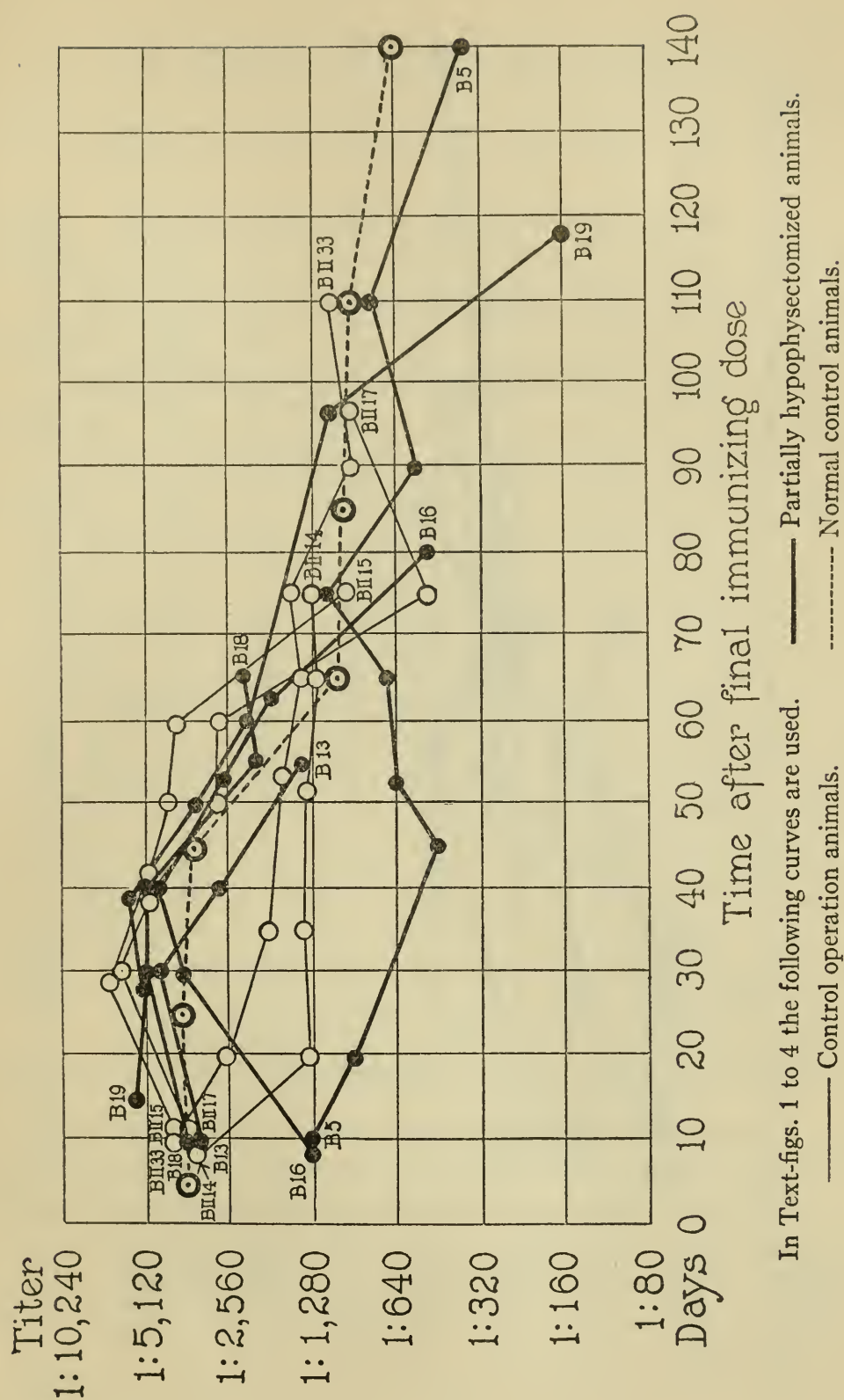
EXPERIMENTAL.

Series 1. The Production of Agglutinins after Hypophysectomy.—Guinea pigs, in which partial hypophysectomy⁶ and a control operation⁶ had been performed were immunized to *Bacillus typhosus* by the above method. Their agglutinin titers were recorded from the 7th to the 10th day after the last injection and followed every week or 2 weeks. Unoperated control animals were carried along in each experimental unit, but their course ran such a comparatively parallel curve to the large number of normal animals studied previously that an average line has been used for Text-figs. 1 and 2 in order to avoid confusion. Text-fig. 1 represents a group of hypophysectomized animals with operated controls compared to the normal average agglutinin curve. The general curve in the hypophysectomized and control operation animals shows a parallelism to the normal controls and needs no comment. The repetition of such experiments brought the same results. Partial hypophysectomy apparently has no influence on the subsequent production of typhoid agglutinins in guinea pigs.

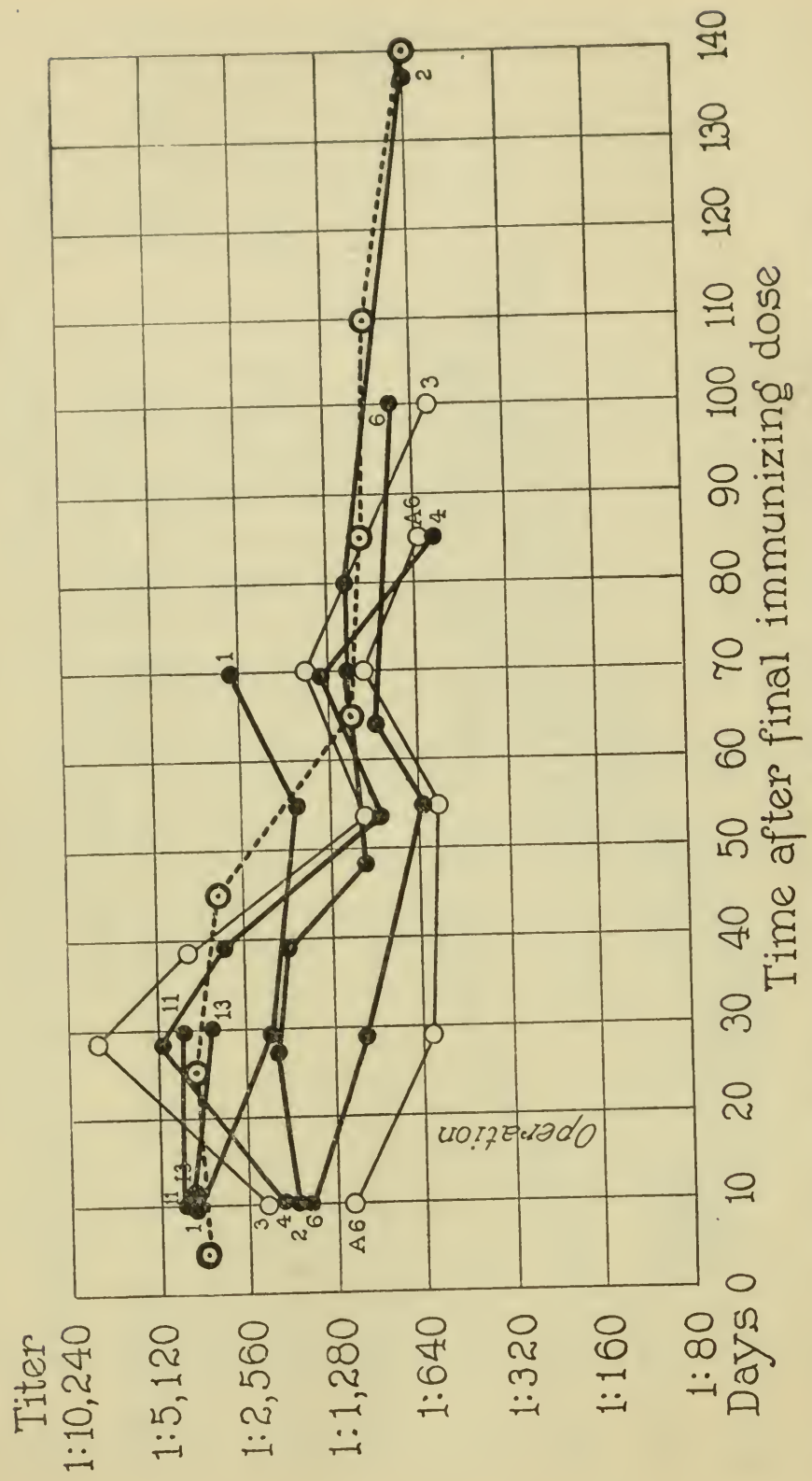
Series 2. The Effect of Hypophysectomy on Antibody Production.—Three sets of experiments were performed. In Class 1 the agglutinin titer in animals immunized to *Bacillus typhosus* was studied before and after hypophysectomy, in Class 2 the hemagglutinin titer, and in Class 3 the hemolysin production. Normal and operated controls were used. For each experimental unit eight animals were used. All were immunized, two were saved for normal controls and three each submitted to partial hypophysectomy or the control operation.

⁵ The guinea pig has a definite menstrual cycle (18).

⁶ At least 8 days after the operation, when the animals were entirely recovered.

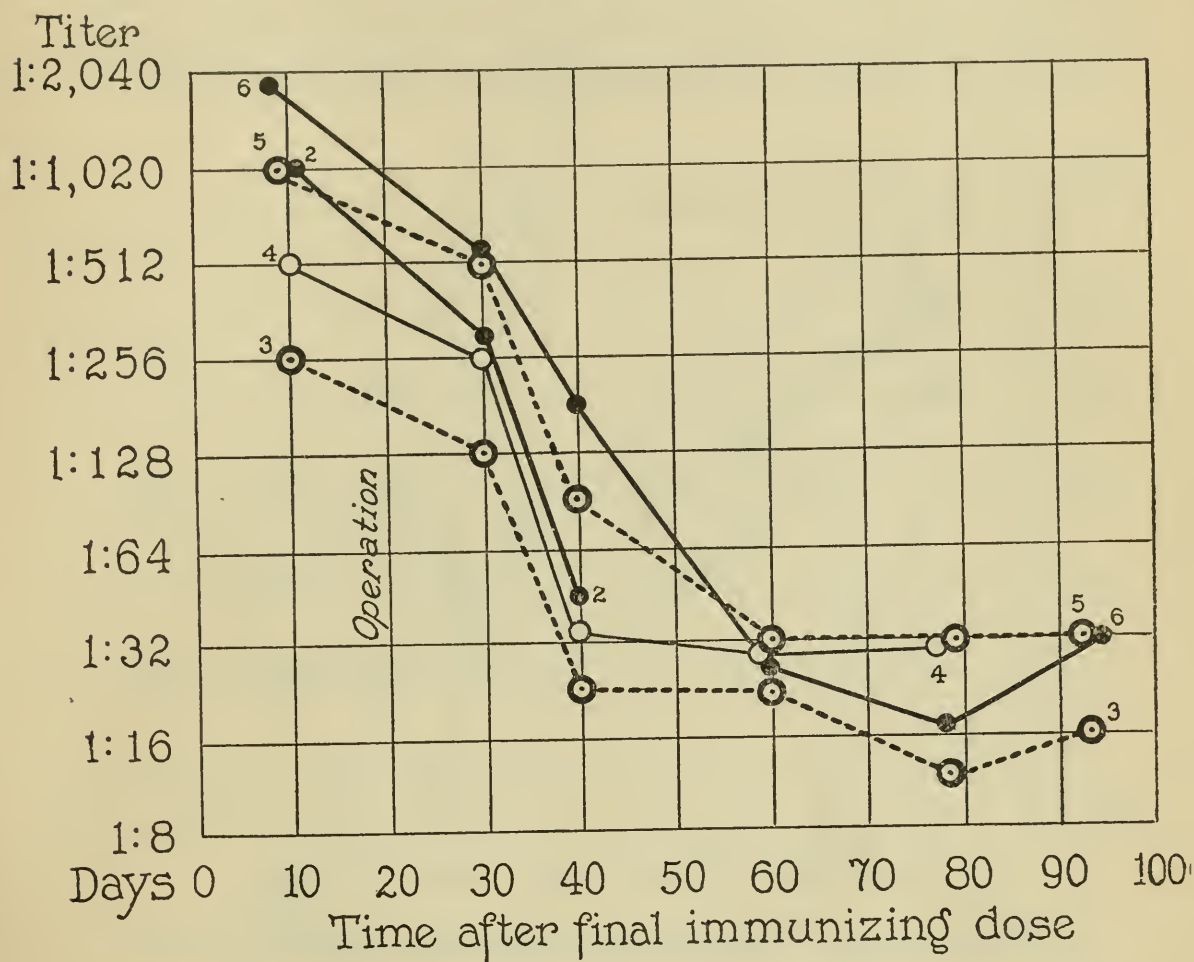


TEXT-FIG. 1. Series 1. Typhoid agglutinin titers of animals immunized after hypophysectomy.



TEXT-FIG. 2. Series 2. Typhoid agglutinin titers before and after hypophysectomy.

Text-fig. 2 represents the study of such an experiment in Class 1, in which the animals were immunized to *Bacillus typhosus*. No special comment is necessary since all titrations fall practically within normal limits.



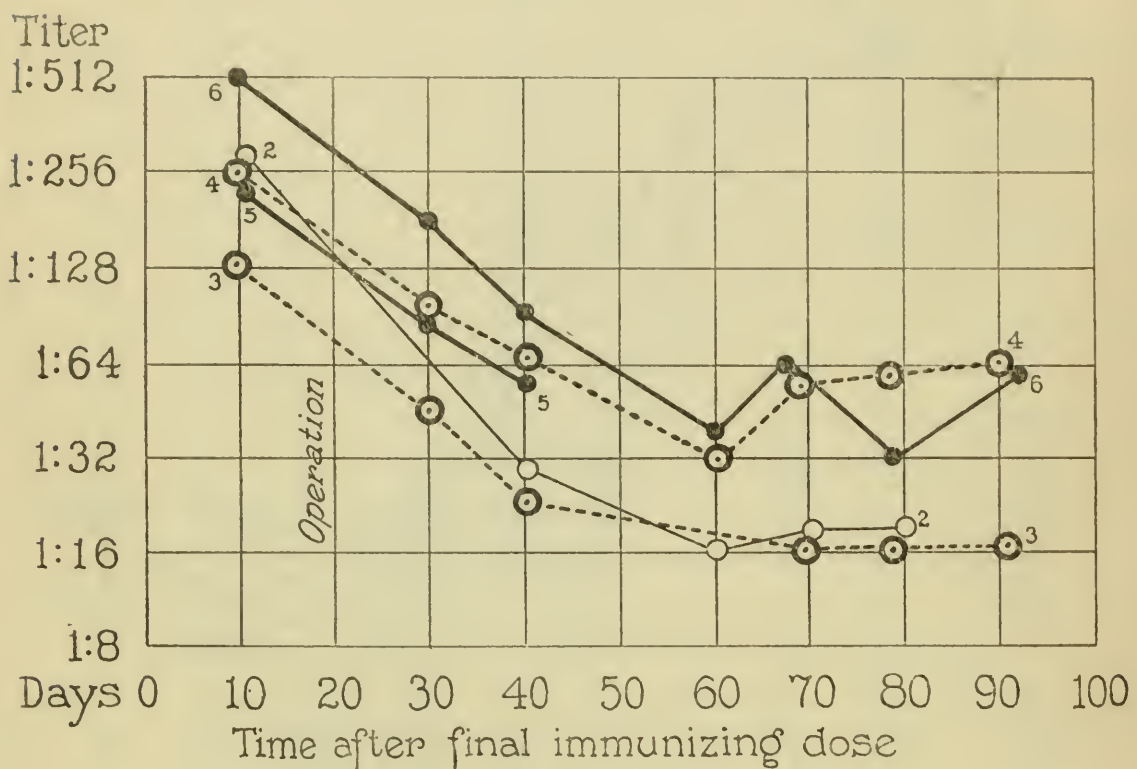
TEXT-FIG. 3. Series 2. Hemagglutinin titers before and after hypophysectomy.

Text-figs. 3 and 4 represent the studies in Classes 2 and 3. Here, too, the parallel of operated animals to normal animals is striking. Repeated experimental units showed no appreciable variation.

These experiments would seem to indicate that partial hypophysectomy has no influence on the maintenance of specific agglutinins for *Bacillus typhosus*, hemagglutinins, or hemolysins in the guinea pig.

Series 3. The Effect of Feeding and Injecting Pituitary Extract on Antibody Production.—In the feeding experiments six animals were

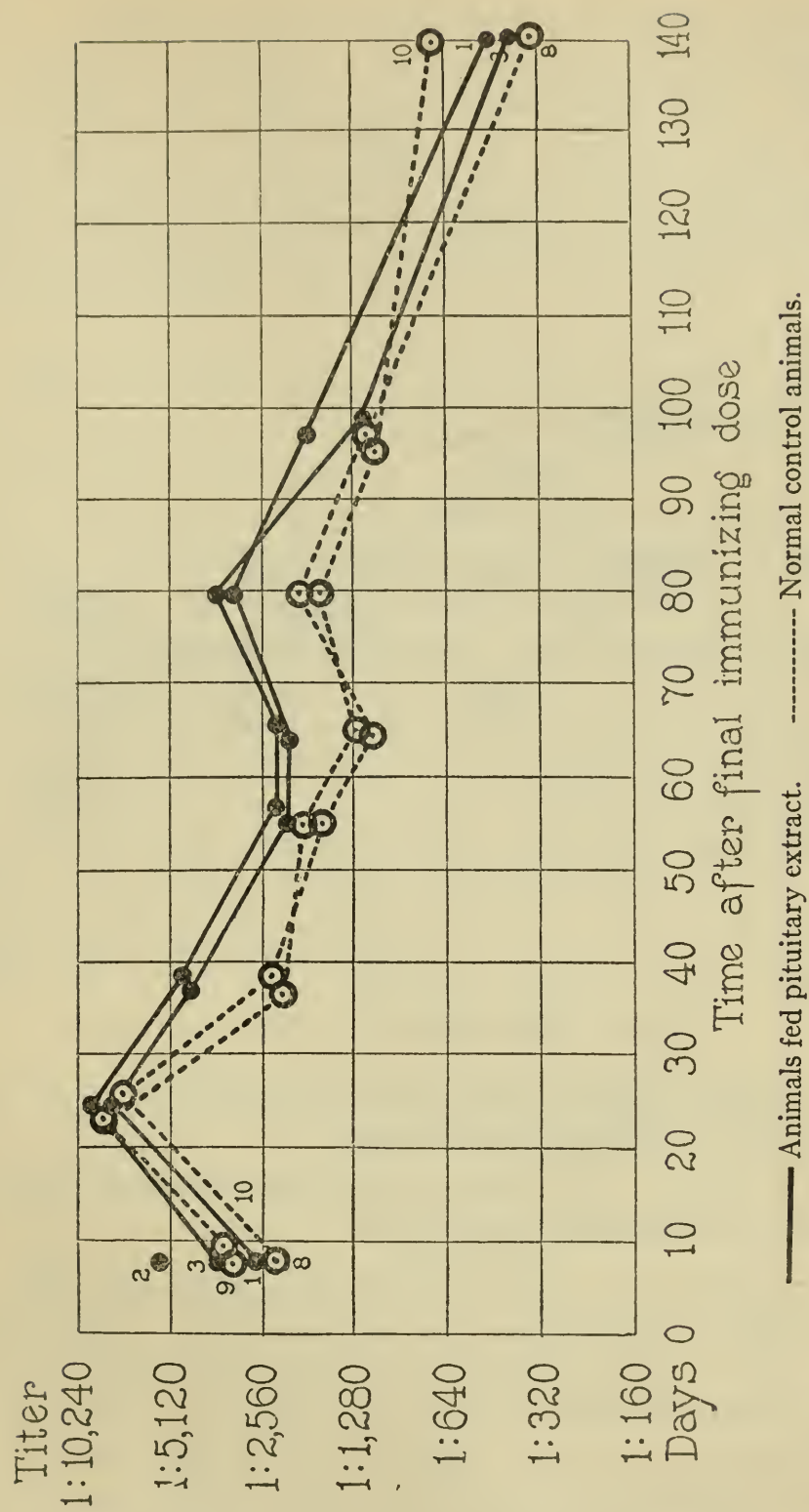
selected and immunization was carried out as described above. Beginning on the day of the first inoculation all animals were fed two medicine droppers full of milk. To the milk of three animals 0.13 gm. of the Burroughs Wellcome whole pituitary gland extract was added. The feeding of milk and of pituitary extract in the same amount of milk was carried out daily until the experiment was finished. This was the method of feeding gland extract adopted by Goetsch (9)



TEXT-FIG. 4. Series 2. Hemolysin titers before and after hypophysectomy.

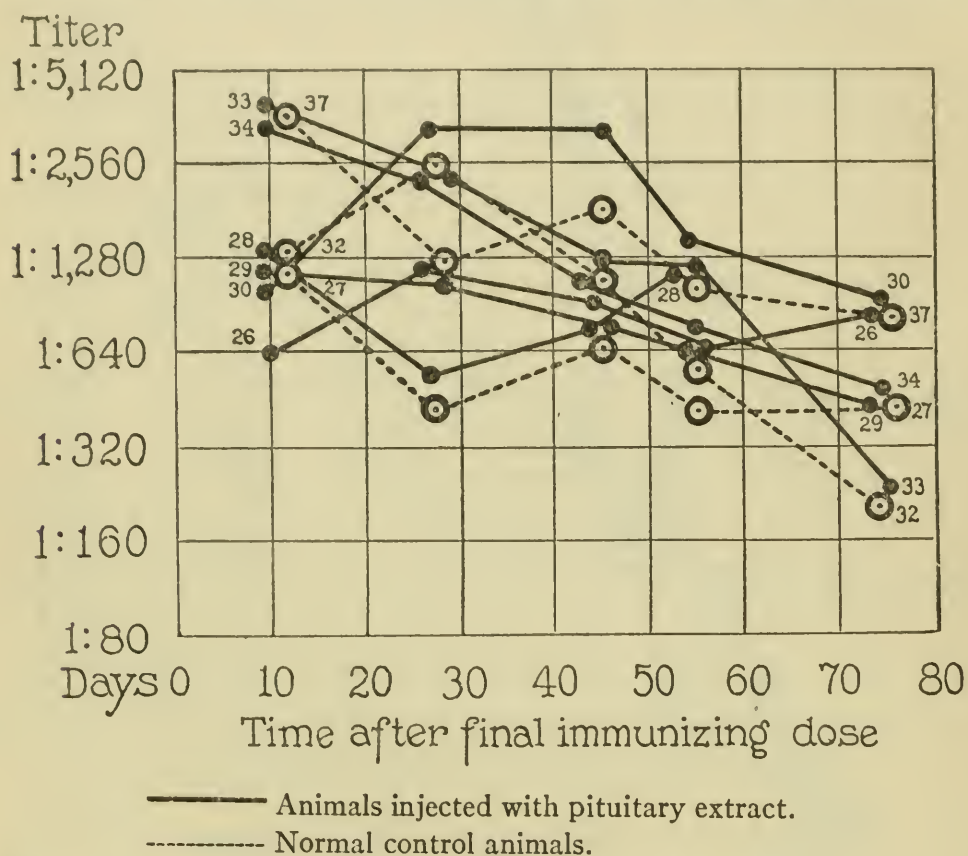
and proved very satisfactory, the animals taking their ration with evident relish. The control animals were fed milk to obviate any criticism of extra nourishment vitiating or influencing the experiments. Beginning 9 days after the last inoculation the agglutinin titer was studied, such studies being then repeated at weekly or biweekly intervals. Text-fig. 5 represents an experimental unit of the above description. The parallelism of normal and pituitary-fed animals is striking.

In another group of animals the gland was given by intraperitoneal injection. 1 gr. of Burroughs Wellcome whole gland extract



TEXT-FIG 5. Series 3. The effect of feeding pituitary extract on the typhoid agglutinin titer. In two animals, only one determination was made because they died from cardiac puncture the day of the first titration.

tablets was used dissolved in 1 cc. of normal saline solution. 1 cc. of normal saline solution was injected at corresponding intervals into the controls. Nine animals were used in these tests, three for controls and six for injection. The animals were first immunized to *Bacillus typhosus*. On the 10th day following the last inoculation their agglutinin titer was determined. This was repeated in 18 days. 10 days later the injection of pituitary extract and salt solution was



TEXT-FIG. 6. Series 3. The effect of repeated intraperitoneal injections of pituitary extract on the typhoid agglutinin titer.

begun and repeated every other day for 3 doses, following which the agglutinin titer was again done. Later the pituitary extract injections were repeated and again subsequent agglutinin titrations carried out. Such an experiment is reported in Text-fig. 6. Further studies varying the amount of extract injected and the intervals between injections were carried out, but no appreciable changes were noticed. As seen in Text-fig. 6 the animals injected with pituitary ran a titer approximate to that of the controls.

SUMMARY.

An operative technique was evolved permitting successful partial hypophysectomy in guinea pigs.

Such animals, when immunized to *Bacillus typhosus*, produced specific agglutinins in the same quantity and at the same rate as unoperated and operation controls immunized at the same time and by the same method.

In guinea pigs previously immunized to *Bacillus typhosus* and hen red blood corpuscles partial hypophysectomy had no effect on the continued production and persistence of typhoid agglutinins, hemagglutinins, and hemolysins.

In guinea pigs immunized to *Bacillus typhosus* both the continued ingestion and the intraperitoneal injection of the whole pituitary gland extract (Burroughs Wellcome) had no effect on the subsequent agglutinin titers as compared to that of normal animals.

The experiments would appear to show either that the hypophysis does not play an important direct or indirect part in the production of and persistence in the blood of typhoid agglutinins, hemagglutinins, and hemolysins, or that the amount of hypophysis left behind in the operation in order to maintain life is adequate also to exercise the degree of functional influence on these processes which the entire hypophysis conceivably exercises.

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STUDIES UPON EXPERIMENTAL MEASLES.

I. THE EFFECTS OF THE VIRUS OF MEASLES UPON THE GUINEA PIG.

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(Received for publication, August 15, 1921.)

Investigations dealing with the nature of the microorganismal excitants of the exanthematous diseases have been numerous during the past two decades, and the present point of view relates the latter to the so called filterable viruses. It is obvious that more precise and definite knowledge of these agents is greatly to be desired.

The nature of the virus of measles has received little attention from the standpoint of research since the transmission experiments of Hektoen¹ showed the existence of the virus in the circulating blood, and those of Goldberger and Anderson² proved that the virus is a filter passer. The recent work of Blake and Trask³ has both extended and confirmed previous knowledge and has appeared at a strategic time when the studies of Sellards⁴ had shaken confidence in the earlier experimental findings.

In the investigation of the virus of measles or of the exanthemata as a whole, it is a striking fact that the ape, chimpanzee, and the monkey especially, have been the experimental animals of choice, and they have been chosen because of the greater likelihood of reproducing in them a clinical picture analogous to that of the human disease.

¹ Hektoen, L., Experimental measles, *J. Infect. Dis.*, 1905, ii, 238.

² Goldberger, J., and Anderson, J. F., The nature of the virus of measles, *J. Am. Med. Assn.*, 1911, lvii, 971.

³ Blake, F. G., and Trask, J. D., Jr., Studies on measles. I. Susceptibility of monkeys to the virus of measles, *J. Exp. Med.*, 1921, xxxiii, 385.

⁴ Sellards, A. W., and Wentworth, J. A., Insusceptibility of monkeys to inoculation with blood from measles patients, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 57. Sellards, A. W., Insusceptibility of man to inoculation with blood from measles patients, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 257; The reaction of monkeys to the inoculation of measles blood, 311.

Since the work of Inada and Ido⁵ on hemorrhagic jaundice, and that of Noguchi on yellow fever, it is evident that the lower type of laboratory animal, namely, the guinea pig, is to be regarded an important host for even highly specialized parasitic microorganisms. The observations of Noguchi⁶ on the effects of the virus of yellow fever in the guinea pig have shown that a detailed study of the temperature, the leucocytes, and the clinical phenomena, comprises in this animal an important basis for establishing the existence of infection. This class of observations may be regarded as having contributed to the discovery of *Leptospira icteroides*. While there is no intention of intimating that a leptospira may be the incitant of measles, it appeared to us that if such phenomena could be induced in the guinea pig by the inoculation of measles virus they would provide a far more practical method of handling the experimental measles problem. The use of guinea pigs in experiments on measles has heretofore been limited, and the results have proved unsatisfactory for the reason that the obvious clinical effects looked for were not detected.

As Hektoen and later other investigators demonstrated the presence of the active agent of measles in the circulating blood during the height of the disease, we chose this medium as the virus material with which to attempt transmission to and propagation in the guinea pig. An abundance of material was available, as, during the months of December, 1920, and January and February, 1921, an epidemic of measles prevailed in New Orleans, providing many suitable cases for observation.

EXPERIMENTAL.

The object of the experiments was the determination of the possibility of transmission of measles virus from man to the guinea pig. If transmission could be successfully accomplished, propagation of the virus by passage from guinea pig to guinea pig and a study of the

⁵ Inada and Ido, A report on the discovery of the causal organism (a new species of spirochæta) of Weil's disease, *Tokyo Ijishinshi*, 1915, No. 1908. Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., The etiology, mode of infection, and specific therapy of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiii, 377.

⁶ Noguchi, H., Etiology of yellow fever. II. Transmission experiments on yellow fever, *J. Exp. Med.*, 1919, xxix, 565.

effect of such passage on virulence, together with a study of the relative infectivity of the passage virus, were next to be attempted. Finally, the plan included the determination of the relative infectivity of blood obtained in various stages of the human disease; namely, prodromal, preeruptive, eruptive, and convalescent.

The blood taken for clinical study was collected under aseptic precautions by means of all glass syringes, immediately placed in sterile flasks containing glass beads, and defibrinated by a rotary motion. Within 15 to 30 minutes after the blood was removed and defibrinated a part was injected into the circulation of the guinea pig. The animal inoculations were made by intracardiac injection of 1 to 2 cc. of material. The guinea pigs were anesthetized with ether and cardiopuncture was accomplished with a No. 24 gauge needle. In order to be certain that the inoculated blood was deposited directly into the heart chambers, removal of a small portion of blood was performed by syringe suction before the introduction of the infecting material.

The method of study after inoculation consisted in twice daily taking of rectal temperatures, daily counting of the total leucocytes, and close inspection of the animals for signs and symptoms of illness during a period of 5 weeks observation. Animals sacrificed at the height of the reaction and those dying subsequent to inoculation were autopsied and a gross and microscopic study of the tissues carried out. Cultures were made from heart's blood and internal organs to determine the presence of intercurrent infection.

The leucocytic count of normal guinea pigs has been made the subject of fairly comprehensive study. Thus, Bethe⁷ gives the average number as 7,240 per c. mm., Kurloff⁸ as 12,600 per c. mm., and more recently Burnett⁹ as 10,897. Hussey¹⁰ is in entire disaccord with

⁷ Bethe, M., Beiträge zur Kenntniss der Zahl- und Massverhältnisse der rothen Blutkörperchen, *Morphol. Arb.*, 1892, i, 207.

⁸ Kurloff, cited by Ehrlich, P., and Lazarus, A., Die Anæmie, in Nothnagel, H., *Specielle Pathologie und Therapie*, Vienna, 1901, viii, 56.

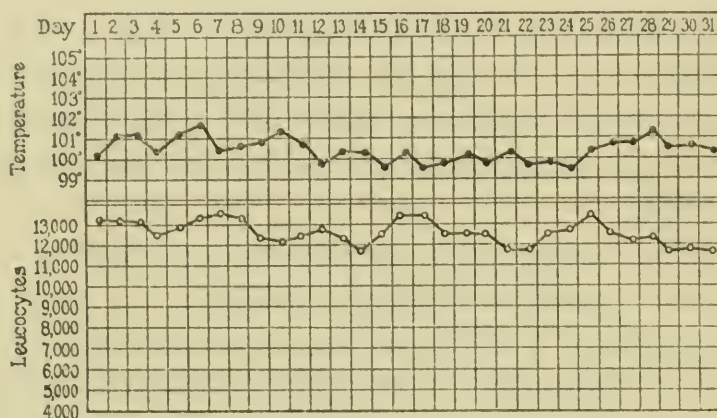
⁹ Burnett, S. H., A study of the blood of normal guinea pigs, *J. Med. Research*, 1904, xi, 537.

¹⁰ Hussey, R. G., General leucocytic response of the guinea pig during the reaction of artificial immunity in experimental tuberculous infection, *J. Exp. Med.*, 1921, xxxiii, 337.

these investigators in that he gives 5,247 as the average count on an unstated number of animals.

We have not been able to find in the literature any extensive reference to the normal temperature limits of guinea pigs. We accordingly decided to construct curves of the rectal temperatures of these animals, in order to furnish a basis for the determination of pyrexia.

Experiment 1 (Preliminary).—Thirty guinea pigs were used in the experiment, temperatures being determined daily for 31 days. For sake of completeness, leucocytic determinations were made at the same time. Text-fig. 1 represents the composite curves constructed as a result of the experiment.

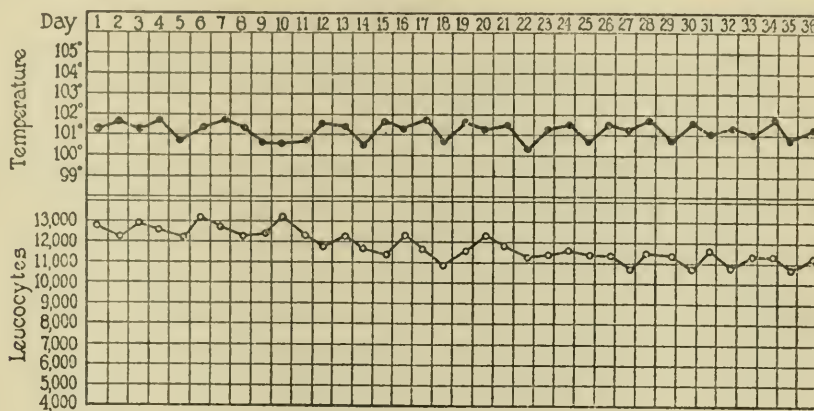


TEXT-FIG. 1. Composite temperature and leucocytic charts of thirty normal guinea pigs.

The results show that the average temperature of the animals in our series, was 100.7°F. , the limits being 100° and 101.4°F. In the main experiments, we disregarded as an elevation of temperature any reading below 102°F. The leucocytes for our series averaged 12,250, the limits being 10,750 and 13,750.

Experiment 2 (Preliminary).—Since defibrinated blood from cases of human measles was to be injected into the circulation of guinea pigs, it appeared desirable to establish whether or not any reactionary objective signs, temperature increase, or leucocytic changes could be induced by the injection of normal defibrinated blood. Accordingly intracardiac injections were performed on a series of fifteen guinea pigs, defibrinated blood from adults and children being given in quantities varying from 0.5 to 5 cc. Temperature and leucocytic curves constructed in Text-fig. 2 show the effects of these inoculations.

It thus appears that normal defibrinated blood when injected into the circulation of guinea pigs occasions no pyrexial reaction or leucocytic changes. Furthermore no objective signs of illness were to be noted in any of these animals. A number of guinea pigs were sacrificed at periods ranging from 7 to 20 days after inoculation and a histopathological study was made of the various organs. In no case were lesions discernible, except small localized areas of congestion and atelectasis in the lungs, to which we paid no special attention, for as pointed out by Maitland, Cowan, and Detweiler,¹¹ such



TEXT-FIG. 2. Effects of intracardiac inoculation of normal human defibrinated blood upon temperature and leucocytes of guinea pigs.

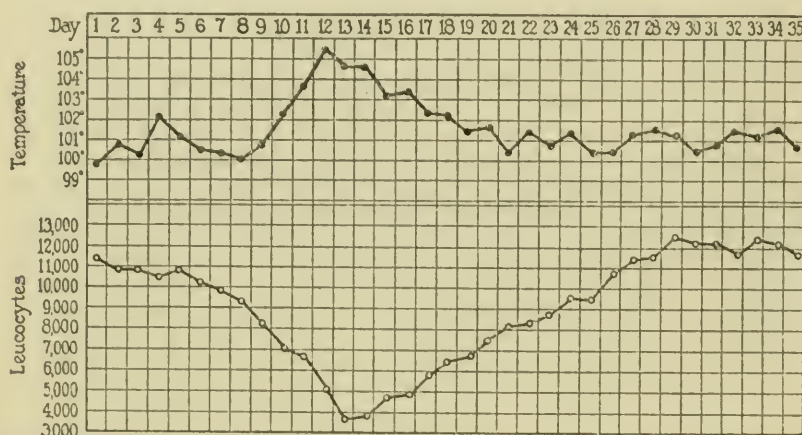
changes are agonal and frequently occur following an anesthetic and death in the guinea pig.

Experiment 3.—The purpose was to determine whether the virus of measles could be directly transmitted from man and propagated in the guinea pig. Blood from seven different cases of measles in the eruptive stage of the disease was employed as the inoculating material. Six of the cases were children ranging in age from 4 to 10 years, and one was an adult. For each experiment of the series, six guinea pigs were used, four for inoculation and two for control. The test animals all received 2 cc. of defibrinated blood containing virus directly into the cardiac circulation, while the controls were given a like quantity of normal blood. In each instance the blood was cultured on ordinary media at the time of injection to exclude bacterial contamination.

¹¹ Maitland, H. B., Cowan, M. L., and Detweiler, H. K., *Brit. J. Exp. Path.*, 1921, ii, 8.

Of the twenty-eight guinea pigs injected with virus, twenty subsequently showed unmistakable reactions, alike in all respects, which could only be interpreted as indicative of transmission of the infecting material. The control animals remained normal throughout the entire period of the experiment. In general, the effect of the blood containing virus upon the guinea pigs was characterized by a gradual rise of from 2–4° in the temperature, beginning on the 11th to 14th days after inoculation, the animals in the interim remaining well to all outward appearances.

The average number of guinea pigs in each of the seven series that gave a definite reaction was three, or 75 per cent. Perhaps a greater percentage of the animals of this series reacted, but we considered as reactions only those which showed a rise in temperature above 102°C. Coincident with the temperature



TEXT-FIG. 3. Temperature and leucocytic curves after inoculation of human measles blood secured at height of eruption.

elevation the leucocytes dropped until a decided leucopenia, as low as 4,000 cells per c.mm. in a number of instances resulted. The fall in the circulating leucocytes was a more constant sign of the reaction than the temperature rise. While in the majority of the reacting animals the temperature rise occurred coincidently with the leucocytic drop, in six animals there occurred a well marked leucopenia by the 12th day with no coincident temperature rise. It is noteworthy that in the majority of the animals reacting there occurred an initial rise in the temperature on the 2nd or 3rd day after inoculation, unaccompanied by any fall in the leucocytes. This pyrexia was maintained only for a period of 24 hours and usually represented an elevation of 1°F. At first we attributed this initial rise to the foreign protein inoculated; however, animals receiving normal blood showed no such rise, and we are unable at present to offer any explanation. Text-fig. 3 graphically represents the typical temperature and leucocytic changes induced.

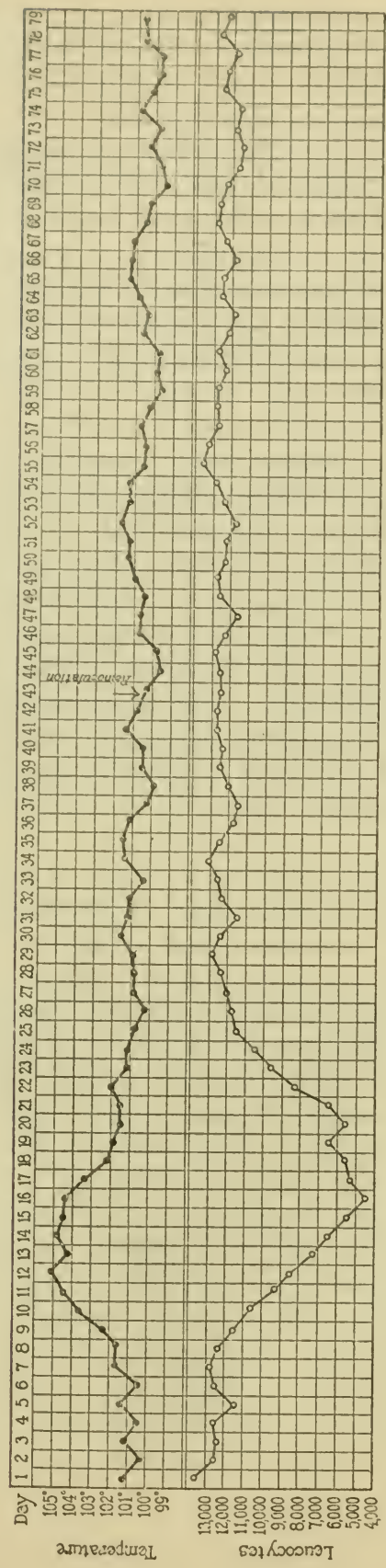
In order to test the effects of reinoculation upon previously reacting animals six recovered guinea pigs were reinoculated with 2 cc. portions of blood secured from human cases of measles at the height of eruption; in none were further pyrexial or leucocytic changes noted (Text-fig. 4).

A number of these animals were sacrificed when the reaction was at its height, and at autopsy the kidney invariably presented microscopic and often gross evidence of acute hemorrhagic nephritis. Blood was removed from certain animals of the series without killing them, and transferred to new guinea pigs for the purpose of virus propagation. Cultural control of the withdrawn blood, which was always carried out, proved negative in all cases for ordinary microorganisms.

Experiment 4.—The purpose was to continue the propagation of the virus by passage from guinea pig to guinea pig. Blood of animals reacting to the direct transfer of measles virus from man was removed by cardiopuncture at the height of the reaction, defibrinated, and injected in 1 cc. quantities directly into the heart chamber of new animals. Seven different passage strains were used and four to six guinea pigs employed. With two strains the transfers were discontinued after the third generation, while with four others the passage was carried to the eighth generation. In these animals the proportion of reactions was greater than with those of Experiment 3. In some series all the guinea pigs reacted in a characteristic manner, in others only part. The reaction was identical with that observed in the direct transmission experiments. After an incubation period of 9 to 10 days there occurred a rather abrupt rise in temperature of 2–4°F. and a coincident fall in the leucocytes. The temperature remained at the peak for 2 to 3 days, gradually subsiding, and reaching normal on the 14th to 16th days after inoculation.

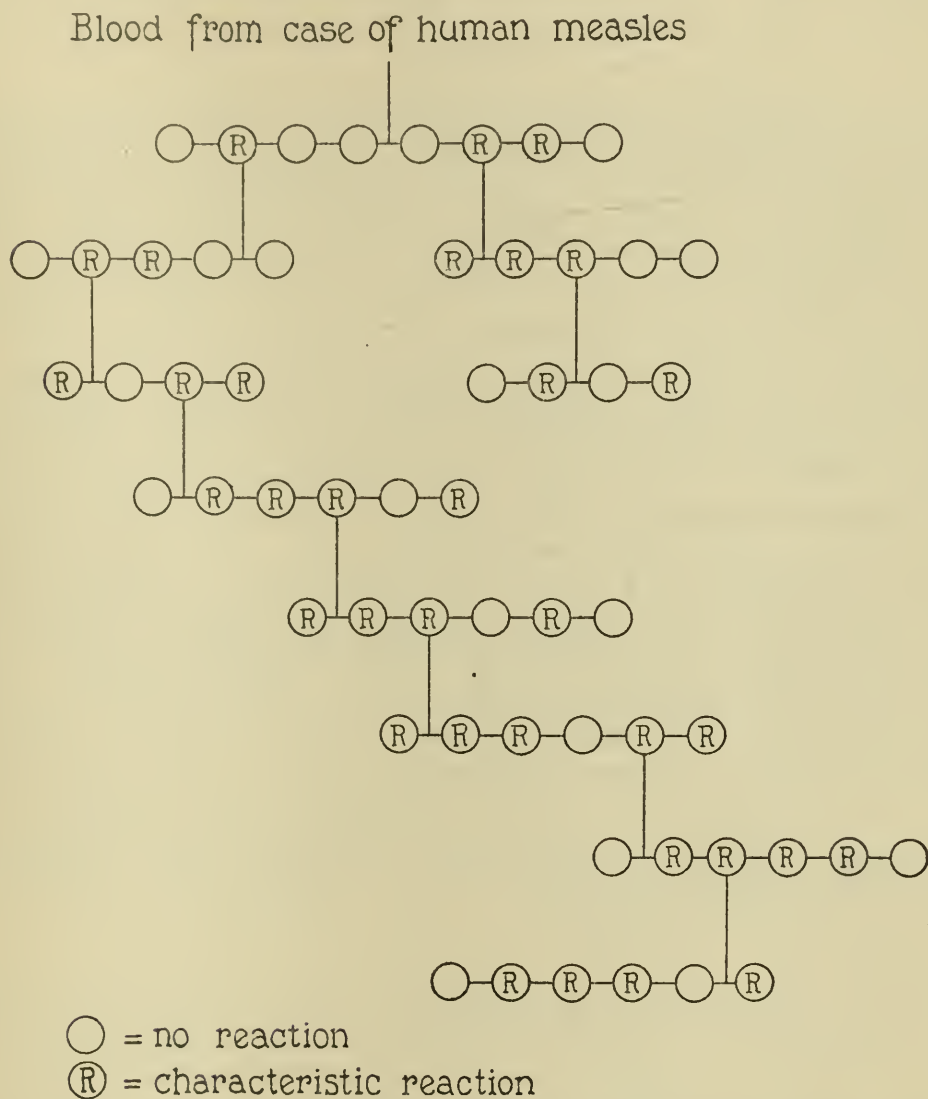
In this experiment it is noteworthy that a number of guinea pigs succumbed presumably as a result of the inoculated virus. Death occurred not infrequently in virus animals of the third generation. Such animals were visibly sick during the period when the reaction was at its maximum; namely, on the 9th to 14th days. They became listless, the hair ruffled, and they remained in the corner of the cage refusing food. Autopsies showed an acute hemorrhagic nephritis which was confirmed by microscopic study. Cultures from the heart's blood, kidney, etc., were always negative. Aside from acute congestion and slight parenchymatous changes, other organs showed nothing worthy of note. The lungs in no instance revealed lesions of any significance. Text-fig. 5 expresses graphically the propagation experiments with a typically reacting passage strain.

Experiment 5.—Since we found that the guinea pig reacted specifically to the intracardiac injections of measles virus, three series of experiments were carried out with a view of ascertaining the relative period of infectivity of the human blood containing virus for the guinea pig. One series of tests comprised twenty-four guinea pigs which were inoculated by the cardiac route with 1 cc. of fresh defibrinated blood that had been secured 1 hour previously from four children exposed to measles by living in close contact with members of their family suffering from



TEXT-FIG. 4. Effects of reinoculation with human defibrinated measles blood secured at height of eruption on temperature and leucocytes of guinea pig which had reacted previously.

the disease. All these children showed 0.5° – 1° rise in temperature and some malaise, and two showed slight coryza. Clinically they were classed in the prodromal stage; later all these children developed measles. Subsequently it turned out that the blood was secured 60, 45, 40, and 26 hours, respectively, before the appearance of the eruption. A second series of twenty-four guinea



TEXT-FIG. 5. Showing the continued reaction in guinea pigs through eight successive generations for a single strain of measles virus.

pigs was injected intracardially with 1 cc. of defibrinated blood from four cases of measles in the preeruptive stage. These cases at the time of collecting the blood showed temperatures ranging between 99° and 100° F. and marked coryza, and two showed Kopliks spots. In this experiment the blood injected was withdrawn from the patients 36, 30, 16, and 12 hours previous to the appearance of

the rash. The third series comprised eighteen guinea pigs which received 1 cc. of fresh defibrinated measles blood secured at periods from 24 to 48 hours after the full abatement of clinical symptoms. It is noteworthy that one of the bloods used was obtained from a case previously tested during the eruptive stage, and from which animal inoculations gave typical leucocytic and temperature reactions.

Animals of Series 1, which were to test the infectivity of the blood in the prodromal stage of measles, did not show at any time an elevation in temperature or decrease in the leucocytes though observed daily over a period of 5 weeks. The negative result in all these animals would indicate that the blood of cases of human measles is not infectious for the guinea pig during the so called prodromal period. As further indication that such is the case, some of these animals were subsequently inoculated with 1 cc. of blood containing virus secured during the eruptive stage, with resulting typical reactions. It is possible, though not probable, that the failure of the animals to respond was due to the small amount of the specific excitant which existed in the quantity of blood injected. We are inclined to believe, however, that the virus had not yet invaded the blood at this stage.

With the second series of animals, the purpose of which was to determine the infectivity for the preeruptive stage, very few reacted, and in none were the reactions very definite. In three out of a total of twenty-four there occurred a slight rise in temperature after an incubation period of apparently 2 weeks, but at no time was there an appreciable decrease in the leucocytes. Two of these animals, however, subsequently failed to react when injected with 2 cc. of blood secured from a human case at the height of eruption. This might be considered as acquired protection in the guinea pig produced by a previous infection. At any rate we conclude from this experiment that the blood of preeruptive cases of measles is not infectious for the guinea pig beyond 12 hours preceding the eruption.

In the third series, in which it was attempted to determine how long the blood of the convalescing human case remains infectious for the guinea pig, none of the eighteen animals inoculated with blood collected 24 hours after the temperature had reached normal, even though the rash was still discernible on the patient, reacted in a suggestive manner.

It would then seem that the infectious phase of the human, virus-containing blood for the guinea pig has a range of approximately 54 hours. This time includes in man the latter part of the preeruptive, and the early part of the temperature declining periods, in addition to the period of temperature height and full eruption.

Experiment 6 (Cultural).—We desire at this time to record very briefly the cultural experiments which were performed during the early part of this study. Blood from cases of human measles and from guinea pigs which had previously reacted to inoculation was defibrinated and cultured on a variety of media. For instance, aerobic and anaerobic cultures were made with red agar, chocolate agar, potato blood agar, and Noguchi media both solid and fluid, as substrate. Quantities of blood varying from 0.1 to 1 cc. were used as the inoculating material,

with incubation at various temperatures for periods ranging from 1 to 25 days. No macroscopic or microscopic growths could be discerned. On a number of occasions we encountered peculiar structures resembling somewhat in appearance the globoid bodies of Flexner and Noguchi.¹² We mention this purely for the sake of completeness, as for the present we are entirely disregarding them and are inclined to look upon them as colloidal aggregates.

SUMMARY.

In general it may be said that three striking alterations occurred constantly in the animals reacting to intracardiac injections of blood from cases of measles; namely, pyrexia, leucopenia, and nephritis. The elevation in temperature usually began about the 9th day following inoculation, the rise being fairly abrupt from the normal to 104°F. and above, at which height it remained with slight fluctuations for 3 or 4 days, finally subsiding by lysis. During the period of fever, particularly in the animals of first transmission, often no objective signs of illness were to be noted, the guinea pigs remaining lively and eating as usual. When the animals showed any outward signs these were manifested by a loss of appetite, lusterless and ruffled hair, and indisposition to move even when disturbed. The typical exanthematous signs of human measles were not observed in any of the animals. In some there occurred a slight coryza and watering at the eyes; however, no special account was attached to these signs as they were rather indefinite and inconstant, especially for animals of first transmission.

Coincident with the rise of temperature there always appeared a fall in the total leucocytic count; in some animals the drop in white cells preceded the rise in temperature by 2 to 3 days. The leucopenia, though well defined, varied for animals of the same series. In some the count was as low as 3,600 cells per c. mm.; in no case did we fail to note at least a moderate fall in the leucocytes. The average time for the appearance of this cellular change was 9 to 10 days following inoculation; in other words, the leucocytic reaction seemed to indicate the end of the incubation period. So constant and striking was the leucopenia that we stress this as the most significant feature of the reaction of the guinea pig to the injection of measles blood, particularly since normal blood produced not the slightest change in these

¹² Flexner, S., and Noguchi, H., Experiments on the cultivation of the micro-organism causing epidemic poliomyelitis, *J. Exp. Med.*, 1913, xviii, 461.

white elements. The greatest leucocytic depression was observed around the 12th to 14th days, which corresponded approximately to the stage of temperature peak. Following the leucopenia, the leucocytic rise was very gradual, taking on the average 8 days to attain the normal level.

The animals killed at the height of the reaction, or those dying presumably from the effects of the blood containing virus, showed as the most constant lesion gross evidences of acute nephritis. The kidneys in these cases were swollen, cloudy, and congested. In certain guinea pigs there were in addition well defined petechiæ and larger blood extravasations scattered throughout the cortical substance. In the animals dying after inoculation, the kidney alterations were found so constant and characteristic that we regarded them as a special index of the experimental infection. In these animals the hemorrhagic areas were not infrequently 2 to 5 mm. in extent, and when occurring on the surface of the organ produced a separation of the capsule. Occasionally free blood was noted in the pelvis, and in the absence of hemorrhage within the pelvic lining, we assumed that it came from the uriniferous orifices of the calices. Microscopically the kidney sections revealed evidence of acute hemorrhagic nephritis, the hemorrhages for the most part being related to the capillaries of the tufts and the tubules of the pyramids. In the most pronounced cases practically all glomeruli were affected. Every stage from marked dilatation of the capillary whirl to well defined intercapillary blood extravasations that often filled the capsular space, and in consequence partially or completely obliterated the glomeruli, were found. Whether a destruction of the endothelium occurred, could not be determined. The absence of a neutrophilic reaction was of special significance, these elements of acute inflammation being nowhere found associated with the kidney lesion. The blood in relation to the tubules commonly occurred in the lumen of the collecting tubules and in the form of red blood cell casts.

The spleen usually was found enlarged, particularly in the guinea pigs presumably dying as a result of the virus injections. Aside from the usual parenchymatous changes common to toxemias, the other internal organs showed nothing of special note.

CONCLUSIONS.

Guinea pigs react specifically to intracardiac injections of defibrinated blood from cases of human measles.

There is a definite and constant rise in the temperature and a coincident decrease in the total number of leucocytes after an incubation period of 9 to 12 days.

The guinea pig reaction is produced with the human blood only during a certain phase of the disease, which period corresponds approximately to the eruptive stage. 36 hours prior to the eruption and 24 hours after the temperature is normal, the human blood gives rise to no reaction in this animal. The reaction follows with greater frequency in animals inoculated with measles blood obtained at the height of the eruption.

Guinea pigs which react and recover are not susceptible to reinoculation with measles blood if tested over periods of 2 weeks to 3 months after recovery.

Guinea pigs receiving normal human blood injected intracardially do not react with leucocytic or temperature changes.

The constancy of the reaction described leads us to conclude that propagation of the virus is obtained by passage of the blood from infected guinea pig to normal guinea pig; that such passage seemingly increases virulence, since a number of animals thus inoculated die during or subsequently to the peak of the reaction. Apparently death in these cases results from acute hemorrhagic nephritis. Ordinary intercurrent or secondary infection plays no part in these effects as shown by careful bacteriological examination.

Postmortem examinations of animals sacrificed at the height of reaction, and of animals dying, presumably, as the result of the specific infection, show an intense hemorrhagic nephritis, which is a constant pathological finding.

Attempts to cultivate the virus of measles so far have been unsuccessful.

A typical exanthem is not noted in the guinea pig following the intracardiac method of inoculation of human blood containing virus. Coryza and injection of buccal mucous membrane occur in some animals, but are so inconstant as not to be included by us as part of the specific reaction.

While the symptoms and lesions produced in the guinea pig are not entirely analogous to those in man, the constancy of a characteristic reaction after a definite incubation period, together with the successful reproduction of this reaction in subsequent guinea pig to guinea pig inoculations, indicates that the virus of measles is capable of being propagated in the guinea pig, and is pathogenic for this animal.

HEMOLYSIS OF ERYTHROCYTES IN CONTACT WITH GLASS.

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In the course of experiments in which it became necessary to make counts of the numbers of red blood corpuscles in various solutions without the use of fixatives it was observed that the cells began to hemolyze as soon as they settled out in the hemocytometer, even though the same cells might remain intact for hours when not in contact with the glass. Hemolysis occurred in some cases so rapidly that it was impossible to make accurate counts. Hemolysis of this sort must have been frequently observed by others, but the literature seems to contain no mention of the fact, nor does any study seem to have been made of the conditions underlying the phenomenon. An investigation of the subject was undertaken with the idea that it might throw some light upon other well known reactions between blood cells and solid bodies, such as the phagocytosis of solid particles, the spreading of leucocytes against solid surfaces, and the disintegration of blood platelets upon contact with foreign bodies.

EXPERIMENTAL.

Contact Hemolysis.

The phenomenon of contact hemolysis may be easily demonstrated in the following manner. A drop of blood is taken from the finger into 25 cc. of 0.9 per cent sodium chloride. The cells are centrifuged out and resuspended in 25 cc. of 0.9 per cent sodium chloride. If a drop of this suspension is mounted on a slide which is not scrupulously clean, the more or less rapid hemolysis of the cells may be readily observed, especially if no precaution has been taken to neutralize the sodium chloride solution which, ordinarily, is as acid as pH 5, even when prepared from chemically pure material. Under

favorable circumstances all the cells may have disappeared in 5 minutes, although there has been no change in the original material. Quantitative data are obtained in the same manner by making frequent counts of the numbers of corpuscles still intact on the slide and in suspension.

TABLE I.

Time Required for 50 Per Cent Hemolysis of Washed Erythrocytes Resting on Glass and in Suspension.

Erythrocytes.	pH	Time required for 50 per cent hemolysis.		
		In suspension.	On glass.	
		hrs.	hrs.	per cent*
Human.	3.5	0.18	0.07	39
	4.0	0.27	0.17	63
	4.0	0.2-0.4	0.10	30±
	5.2	1-2.+	0.60	50-
	6.0	3.+	2.+	—
	9.3	2.90	1.10	38
	10.0	2.+	0.87	50-
Rabbit.	3.3	0.20	0.07	35
	3.5	0.32	0.07	22
	3.6	0.36	0.10	28
	3.8	0.52	0.05	10
	4.0	0.31	0.06	19
	4.2	0.6-1.0+	0.15	25-
	9.6	0.43	0.22	51
	9.7	0.32	0.22	69
	9.8	0.37	0.23	62
	9.8	0.30	0.24	80
	10.05	0.40	0.44	110
Average.....				47

* Time on glass in per cent of time in suspension.

In some cases the hemolysis under the cover-slip was quite irregular in different parts. This is indicated in the tables either by two figures (as 0.26-6.0), showing the approximate extent of the irregularity, or by a minus sign, indicating that the counts were made where hemolysis was slowest.

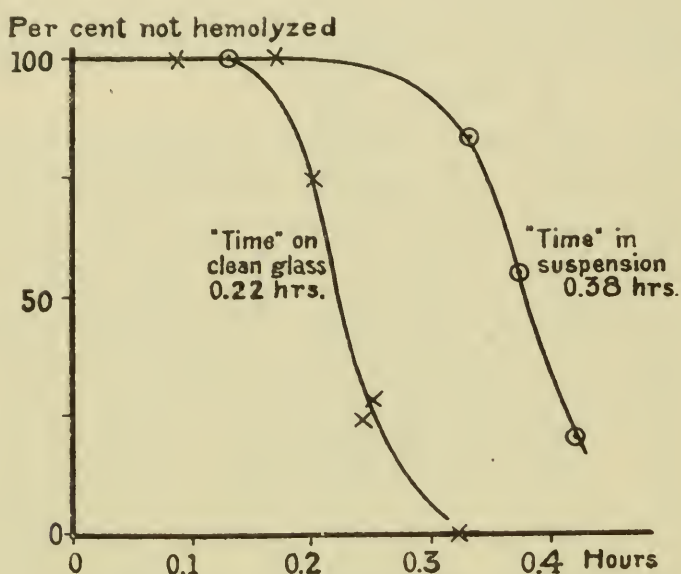
The results of eighteen such experiments with washed rabbit and human erythrocytes are given in Table I. In using human blood, approximately 0.01 cc. was taken from a finger-tip in a calibrated capillary pipette, washed once in 25 cc. of 0.9 per cent sodium chloride, and resuspended in a similar volume of isotonic salt solution. Mix-

tures of varying pH were prepared by taking 5 cc. of this suspension (in some experiments only 1 cc.) plus 1 to 5 cc. of 0.004 M sodium hydroxide or 0.005 M hydrochloric acid in 0.9 per cent sodium chloride plus enough 0.9 per cent sodium chloride to make 15 cc. The pH was measured by a Bovie hydrogen ion potentiometer and a Clark hydrogen electrode. In experiments on rabbits, 0.3 cc. of blood was taken from the ear vein of a rabbit into a pipette previously rinsed in a 0.5 per cent solution of sodium oxalate in 0.9 per cent sodium chloride to delay clotting, centrifuged in 50 cc. of 0.9 per cent sodium chloride, and resuspended in a similar volume of salt solution. Mixtures were then prepared as for human blood, with the exception that usually only 1 cc. of cells was used.

To determine the rate of hemolysis on glass, a cover-slip was supported on the slide at four points by fragments of another cover-slip; and the mixture was allowed to run under the cover-glass, which was then sealed with paraffin. Counts were made at once over equal areas as measured by an ocular micrometer disc ruled in squares. Frequent counts were made during the experiment, the points on the slide counted being chosen more or less at random in order to average to some extent the variations sometimes observed even under the same cover-slip. The course of hemolysis in the remainder of the mixture, left in suspension in the test-tube, was followed simultaneously. It was thought advisable, for the sake of uniformity, to do this by counting the number of intact corpuscles in a given volume on a hemocytometer. Ordinarily about 200 cells were counted for each determination. The results were then calculated in per cent of the original, plotted on coordinate paper, and the time necessary for 50 per cent hemolysis was determined graphically. Text-fig 1. shows two typical curves having the characteristic S shape of hemolysis curves. The time is determined most accurately at the 50 per cent mark, the point of most rapid hemolysis.

For "clean glass" experiments the slides were first thoroughly washed by the laboratory assistant by the usual methods, including treatment with soap and water, cleaning solution, and finally drying with a clean towel. For uniformity they were again allowed to remain in cleaning solution for a time, were rinsed in hot running tap water for 10 to 20 minutes, then in boiling distilled water, and were

finally stacked on a glass rack where they dried in less than a minute. During the final stages of the process they were handled only with glass tweezers. This method has the advantage of avoiding the grease which inevitably collects on the cleanest towel and it saves the labor of drying. It is easy to demonstrate the presence of what is probably a film of grease on a slide by scraping the surface gently with a pair of tweezers held perpendicularly. On a perfectly clean slide a distinct squeak is heard. On slides cleaned by the usual



TEXT-FIG. 1. Curves showing the percentages of washed rabbit erythrocytes still intact at various times after mixing. The time necessary for 50 per cent hemolysis of these cells in suspension (pH 9.8) was 0.38 hours, while the hemolysis time for the same cells resting on glass was only 0.22 hours.

methods involving wiping, there is usually sufficient grease to act as a lubricant so that the tweezers move smoothly over the surface.

The figures in Table I show that hemolysis of washed human and rabbit erythrocytes which are resting on clean glass occurs in less than one-half the time necessary for the cells in suspension. That this cannot be due to small amounts of alkali is shown by the fact that the phenomenon occurs at the point of acid hemolysis as well as at the point of alkali hemolysis. It is, if anything, more pronounced in acid solutions.

Since it was most natural to connect this phenomenon with the clotting of blood on contact with foreign bodies and the disintegration of blood platelets, it seemed advisable to see whether the disintegration of the erythrocytes could be prevented by a thin film of vaseline, paraffin, or paraffin oil on the slide. In Table II the hemolysis time of washed human erythrocytes in suspension is compared with the hemolysis time on a slide covered with a thin film of vaseline. The vaseline was taken from a solution in ether used in the laboratory for coating cannulas to prevent clotting. As on clean glass, the erythrocytes hemolyze more than twice as rapidly on vaseline as they do in suspension. Many of the experiments on vases-

TABLE II.

Time Required for 50 Per Cent Hemolysis of Human Erythrocytes on a Vaselined Slide and in Suspension.

pH	Time required for 50 per cent hemolysis.		
	In suspension.	On vaseline.	
	hrs.	hrs.	per cent
3.3	0.20	0.07	35
3.5	0.18	0.10	55
4.0	0.27	0.17	63
6.0	3.+	2.+	—
10.0	2.+	0.60	30—
10.3	1.+	0.37	37—

line were done simultaneously with experiments on clean glass, with the same mixtures. In such instances, when a direct comparison between vaseline and glass was possible, no significant difference between the two was found. The film of vaseline was just visible to the naked eye; and the microscope revealed many small drops of vaseline, which rounded up in contact with the water and sometimes became hardly distinguishable from blood corpuscles.

Table III gives the results of another experiment with washed rabbit corpuscles in which the time of hemolysis on paraffin and paraffin oil was compared with that in suspension. The paraffin oil ("highest purity"), like the vaseline, offers no protection against hemolysis. The results with paraffin are more irregular, but here too, in most

cases, hemolysis is more rapid in the sedimented cells than in those suspended. There seems to be some other factor involved in the case of paraffin, however, and to some extent with paraffin oil, because the time curves of hemolysis have frequently an abnormal shape, indicating that hemolysis begins very rapidly and stops short of completion, leaving part of the cells intact, sometimes for hours, after all the suspended cells have disappeared. The reason for this abnormality is not apparent. The paraffin or paraffin oil was applied to the slide with a clean cloth, thus producing a thin film barely vis-

TABLE III.

Time Required for 50 Per Cent Hemolysis of Rabbit Erythrocytes on Films of Paraffin and Paraffin Oil, and in Suspension.

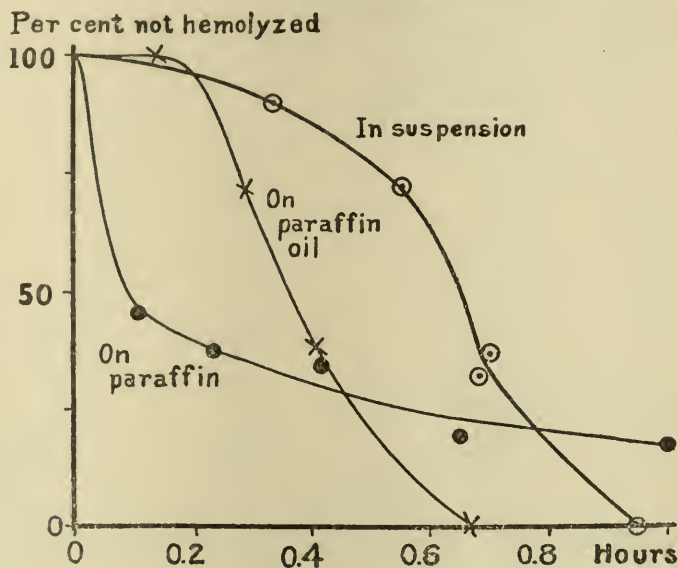
pH	Time required for 50 per cent hemolysis.				
	In suspension.	On paraffin.		On paraffin oil.	
	hrs.	hrs.	per cent	hrs.	per cent
3.3	0.20	0.14*	70	0.08*	40
4.0	0.44	0.26-6.0	59+	0.10	23
4.0	0.45	0.12	27	0.14	31
4.2	6.0	6.00	100	0.60	10
9.7	0.56	0.13	23	0.25-0.35	45-53
9.7	0.68	0.09-	13-	0.36-	53-
10.3	0.38	0.38	100	0.18-	47-
11.05	0.15	0.10	67	0.04	27
11.4	0.07	0.03-0.08	42+	0.05	70

* Not completely hemolyzed until after the control, the shapes of the curves being different as explained in the text.

ible to the naked eye but very evident under the microscope—as ridges in the case of paraffin or as drops in the case of paraffin oil. A typical experiment of this series is plotted in Text-fig. 2.

While contact hemolysis does occur with clean glass, it is much more pronounced with slightly soiled glass. Text-fig. 3 represents the results of a series of experiments in which the hemolysis of rabbit erythrocytes on paraffin, paraffin oil, clean glass, and in suspension is compared with the time necessary for hemolysis of the same cells in contact with a soiled slide. The slides were first washed clean by

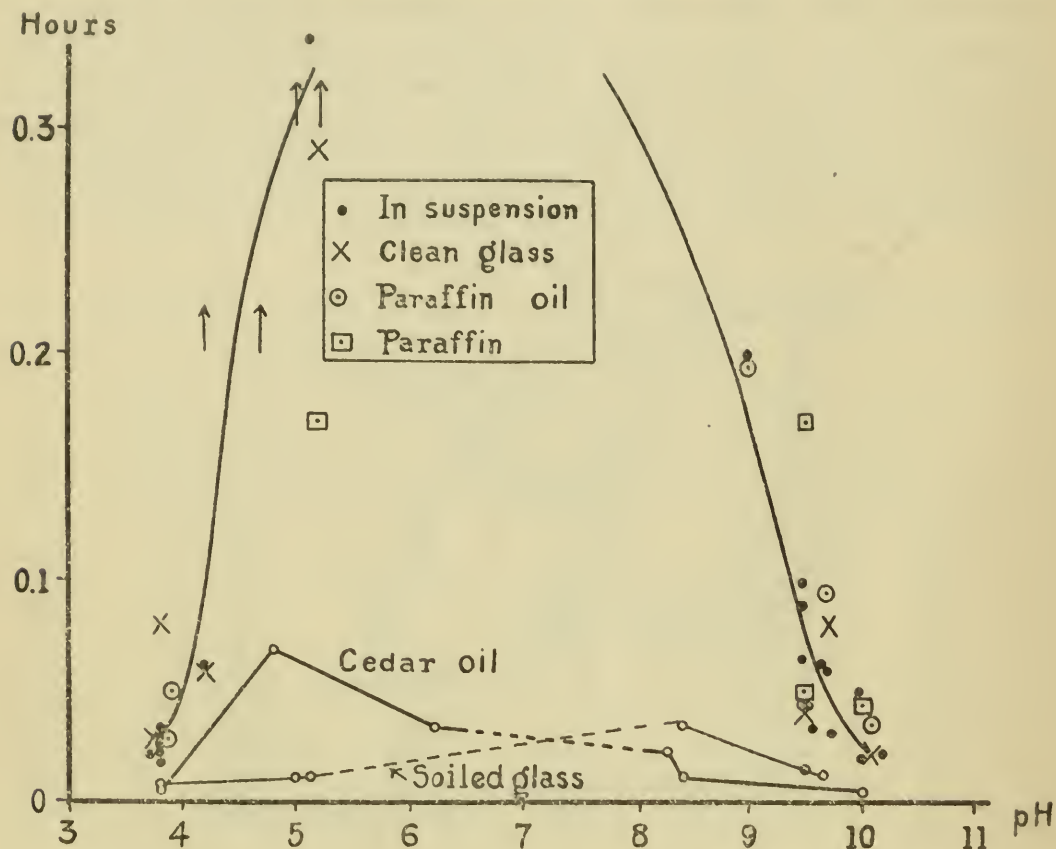
the usual method and were then wiped with a cloth which had been rubbed once on the hair but which was otherwise clean. This treatment produced a dimly visible film on the surface of the glass. The extremely rapid hemolysis is very evident from the figure. A similar experiment, with slides coated with a specimen of cedar oil used for immersion lenses also shows abnormally rapid hemolysis. There was no assurance of the purity of this oil, however, and the result is not of great significance. This figure shows that there is no constant difference between the rate of hemolysis in suspension and the rate of hemolysis on glass, paraffin, or paraffin oil. The apparent dis-



TEXT-FIG. 2. Curves showing the rate of hemolysis of a suspension of washed rabbit cells (2,200 per c. mm.) at pH 9.7 resting on films of paraffin and paraffin oil, and in suspension.

crepancy between this experiment and those previously cited is due to the serum content of the mixture, since the cells were not washed before use. Allowing 40 per cent for the volume of the corpuscles in whole blood, it may be calculated that the serum content of the unwashed cells in the final mixture (5 cc. of cells to 10 cc. of hydrochloric acid or sodium hydroxide) was 0.12 per cent of the normal serum content of blood. Even with this small concentration of serum, contact hemolysis is prevented to a considerable extent on clean glass, though in some experiments it was still evident, particularly in acid solutions. The results of all available comparisons on

clean glass are given in Table IV. In not a few instances the cells on the glass hemolyze even less rapidly than those in suspension. Comparison with Table I reveals the striking effect of small amounts of serum in hindering contact hemolysis.



TEXT-FIG. 3. Curves showing the time necessary for 50 per cent hemolysis of rabbit erythrocytes in 0.12 per cent serum in solutions of various pH values. The rate of hemolysis in suspension is compared with the rate of hemolysis when the cells were resting on clean glass or glass covered with films of paraffin, paraffin oil, cedar oil, or dirt obtained by rubbing a clean cloth on the hair. Arrows indicate that the hemolysis time was greater than the value indicated. Figures showing the hemolysis time in suspension and on clean glass under these conditions are given in Table IV.

In some of the experiments with serum, unwashed cells were used; while in others an equivalent amount of serum was added to washed cells. No alteration in behavior due to the washing was detected.

Further experiments would be desirable in order to see whether the effect of serum is due to the calcium content and whether it can

be destroyed by heating. One experiment was tried to make sure that it was not due to the small amount of oxalate present from rinsing the pipette before taking the blood, but no change in the rate of hemolysis was detected from twice the usual amount of oxalate in the same volume of sodium chloride without serum. In one experiment 0.02

TABLE IV.

Time Required for 50 Per Cent Hemolysis of Rabbit Erythrocytes in 0.12 Per Cent Serum on Clean Glass and in Suspension.

pH	Time required for 50 per cent hemolysis.		
	In suspension.	On glass.	
	<i>hrs.</i>	<i>hrs.</i>	<i>per cent</i>
3.3	0.24	0.08	33
3.3	0.23	0.15	65
3.4	0.23	0.15	65
3.8	0.40	0.27	68
3.8	0.35	0.27	77
3.8	0.22	0.5-1.0	228+
3.8	0.28	0.28	100
3.8	0.25	0.22	88
4.0	0.17	0.26	153
4.0	0.18	0.22	120
4.0	0.22	0.27	122
4.2	2.+	2.+	—
4.2	0.64	0.6	94
4.7	2.+	0.8-1.2	50—
5.2	3.+	2.8	90—
6.4	1.+	1.+	—
9.5	0.44	0.43	98
9.7	0.54	0.80	140
9.7	0.42	0.42	100
9.8	0.36	0.36	100
9.9	0.24	0.23	96
10.0	0.35	0.41	117
10.2	0.20	0.25	125

per cent of serum was found to prevent hemolysis on glass almost completely, which otherwise took place in about half an hour. This was probably largely due to the buffer action of the serum. No measurements of the pH were made in this experiment.

In a few preliminary experiments it was found that 2 per cent dextrose and 0.5 per cent acacia definitely inhibited hemolysis of human

red cells at pH 5.2, while 0.25 per cent acacia failed to do so. More determinations are necessary to establish this as a fact. Interest in these substances arises from the work of Rous and Turner (1), who recommend dextrose-Locke's solution for preserving red cells and gelatin to protect them from injury during centrifuging. Acacia might be expected to resemble gelatin in this respect.

In view of the hemolyzing effect of small amounts of impurities on the glass it is evident that the interpretation of the results is always open to the possible but improbable objection that the glass was not perfectly clean. It was not thought practicable, however, as a routine measure in such a large number of determinations to subject the glass to more extended treatment, such as rubbing with grease-free diatomaceous earth, etc. It is evident, nevertheless, that contact hemolysis is a phenomenon which must occur frequently in experiments on the preservation of red blood corpuscles *in vitro* (1). Also, the hemolysis often observed in centrifuging washed red blood corpuscles is doubtless of a similar nature. In all such experiments it is essential that the pH be controlled more accurately than has usually been considered necessary, since the hemolysis limits are much narrower for cells in contact with glass. The usual acidity of chemically pure preparations of sodium chloride (pH 5.0) and the alkalinity of sodium citrate solutions make this particularly advisable.

A number of experiments were tried with fresh mica surfaces, in the hope that they might be found cleaner than paraffin or glass, but the result was even more irregular. In some areas on the slide the cells hemolyzed much more rapidly, in others somewhat less rapidly than the control cells in suspension. On the whole, it may be said that contact hemolysis occurs on mica as well as on the other surfaces used.

In explaining these results three possibilities are open. In the first place, one thinks of soluble materials coming from the glass. Thus Hausmann and Kerl (2), in studying oligodynamic hemolysis by various metallic plates, observed that hemolysis occurred also around a glass rod left in the plate of agar containing the red blood corpuscles, and attributed the results to alkali given off by the glass. This explanation, however, seems excluded here because of the fact that contact hemolysis occurs in acid as well as in alkaline solutions.

Another proposal is that of Schwyzer (3), who believed that the formation of rolls by red blood corpuscles in contact with glass was due to a neutralization of their electric charge. This explanation is also somewhat improbable in view of the fact that contact hemolysis occurs on both sides of the isoelectric point of the red blood corpuscles (pH 4.7, as given by Coulter (4)).

A more useful working hypothesis seems to be that contact hemolysis is the result of an attempt on the part of the corpuscle or, perhaps, some ingredient of the corpuscles, as the lipoids, to spread on the glass by surface tension, thus accelerating the disintegration of the structure. It may be supposed that this spreading would occur in neutral as well as in acid and alkaline solutions if it were not for the more or less rigid structure of the corpuscle.¹ This conception is readily correlated with the experiments of Bechhold (5), who observed that when red blood corpuscles were shaken with clay or kieselguhr, the particles of the latter could be observed sticking to the outside of the cells. On shaking the mixture vigorously enough to detach these particles, hemolysis occurred. His interpretation was that the particles tore away with them parts of the cell (he suggests lipoid), thus serving to break up the intimate association of lipoid and protein upon which he conceives the rigidity of the corpuscle to depend. The rigidity of the cells, which keeps them disc-shaped, is obviously reduced in alkaline solutions when they round up. During acid hemolysis on the glass the cells are larger in diameter, either because of swelling (Price-Jones (6)) or because they spread out on the glass into a hemispherical shape. Simultaneously they become lighter in color and focus the rays of light at a higher point than do normal cells. This may be due to a more or less hemispherical shape (such as obtains in a cell which spreads out), or to a lower refractive index, or to both.

If this hypothesis were true, the erythrocytes might be expected to stick to the glass most firmly in those solutions in which they show contact hemolysis to the greatest extent. From a few experiments on the stickiness of erythrocytes, which are described later, it may be

¹ This seems somewhat more probable than the alternative assumption that the cells are not pulled out by surface tension at neutrality as strongly as in acid or alkaline solutions.

concluded that the cells are more sticky in acid solutions and less so in alkaline than they are at neutrality. This observation clearly supports the idea that contact hemolysis, in the acid range at least, is due to a spreading out of the cell. If the same conception is to be applied to alkaline solutions in which hemolyzing cells are, if anything, smaller than normal and in which their stickiness is also less, it must be assumed that some ingredient of the cells, as the lipoids, is spreading invisibly. Since there is as yet no experimental basis for this idea, speculation is futile. It may be pointed out, however, that the flotation processes for the separation of different ores depend in general upon the greater tendency of certain particles of ore to stick to oil drops, suggesting by analogy a greater attraction of the glass for lipoids than for other parts of the cell. However that may be, some readjustment of the cellular equilibrium would be expected, from ordinary principles of surface tension, when a complex system of different phases like a red blood corpuscle comes into contact with a solid body. While this readjustment may not have a detectable effect on a normal cell, it may well accelerate disintegration of a cell which is already at the point of acid or alkaline hemolysis.

It is possible that differences in the sensitivity of erythrocytes to contact with glass may occur between species or between individuals, though no attempts have been made to detect them. The method, however, is not very well suited for the measurement of small variations. One is always confronted by the possibility of invisible films of impurities on the glass which destroy the constancy of the results.

Experiments on Stickiness of Erythrocytes.

Experiments on the stickiness of erythrocytes were undertaken by two methods. In the first, the erythrocytes were allowed to settle out in a vessel made by sealing, with paraffin, a short glass cylinder, 2 cm. in diameter and 3 cm. long, to a microscope slide, the other end being closed by a rubber stopper with a capillary air vent. After an hour or two the vessel containing the supernatant solution and the sedimented cells was rotated on a motor ten or fifteen times in as many seconds about a horizontal axis, thus permitting the circulating liquid to wash off a certain proportion of the cells. This proportion was determined by counting the cells washed off and those still sticking on in known volumes and areas respectively.

The effect of pH on the stickiness of washed rat erythrocytes as measured by this method in two experiments is shown by the following figures.

pH	Erythrocytes stuck on slide.	
	A	B
	<i>per cent</i>	<i>per cent</i>
5	17	15
7	0	9
9	0	0

The H ion concentration was maintained by buffer solutions of phosphates in $\frac{M}{60}$ solutions. At pH 5, in both cases, many hemolyzed cells, which had not been washed off when the vessel was rotated, could be dimly seen on the slide. This shows that after contact hemolysis in acid solutions the remains of the cells are stuck to the slide and indicates that contact hemolysis in acid solutions may be due to a spreading of the cells.

In another similar experiment, in which there was apparently no hemolysis, no cells were found sticking to the slide at pH 7 or 9, while there were 700 per sq. mm. at pH 5.

Further measurements by the same method with varying concentrations of serum indicate again an apparent relation between contact hemolysis and stickiness. The results were as follows:

Serum.	Erythrocytes.		
	Washed off.	Stuck on slide.	Remainder, hemolyzed.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.00	43 \pm 9	10	47
0.17	100 \pm 6	0	0
0.35	83 \pm 7	0	17
0.70	85 \pm 7	0	15

In the presence of serum, no cells stick to the glass and there is no appreciable hemolysis (0, 17, and 15 per cent). Without serum, 57 per cent of the cells are either stuck to the slide or hemolyzed, hemolysis being probably the end-result of sticking. The solution

of 0.9 per cent sodium chloride, used to dilute the serum, was carefully neutralized. Hemolysis was doubtless accelerated by contact with the glass, which lasted in this instance for $1\frac{1}{2}$ hours at 25°C .

The other method by which the stickiness of erythrocytes was measured consisted in allowing a suspension of red blood corpuscles in the solution under investigation to run under a cover-slip, which was supported at four points on a microscope slide by small fragments of a cover-slip, as in the experiments on contact hemolysis. The edges of the cover were sealed with paraffin. After a certain interval of time (5 to 15 minutes) the preparation was inverted and the percentage of cells which were stuck to the slide firmly enough to prevent them from falling off was determined by counting the numbers of cells observed over equal areas on cover and slide. In acid solutions almost no cells drop off no matter how long a time is allowed.² In alkaline solutions somewhat variable results are obtained, depending upon the time intervals allowed before and after inverting the preparation. This variation is probably due to the fact that it takes some time to reach equilibrium in alkaline solutions. If the suspension is mounted as soon as mixed and inverted as soon as the cells have settled out, it may take 15 minutes or an hour for the cells to drop off. If, however, 5 minutes are allowed between the time of mixing and the time of mounting, or if 15 minutes are allowed between the time of mounting and the time of inverting, a large proportion of the cells usually drops off during the first 5 minutes after inverting. Two series of experiments by this method are summarized in Table V. In Experiment A, the cells were left in suspension 5 minutes after mixing, to reach equilibrium, and the counts were then made within 5 minutes after inverting. In Experiment B, the cells were mounted immediately after mixing and inverted within 5 minutes, but counts were not made for 15 minutes after inverting. To avoid further complications, this study was limited to those cases in which hemolysis had not begun during the time of the observations. The pH

² The behavior of erythrocytes in this respect reminds one strikingly of the device used by Perrin in determining Avogadro's constant by observations on a gamboge emulsion. Acidification of the solutions caused the globules of gamboge to stick to the walls of the vessel, where they could be easily counted (Perrin, J., *Die Atome*, Dresden, 1914).

values were determined electrometrically and were varied by the addition of 0.005 M solutions of sodium hydroxide and hydrochloric acid in 0.9 per cent sodium chloride. The cells were obtained by centrifuging a few drops of blood from a pricked finger in a known volume of salt solution. When the cells were not washed, the concentration of serum present was calculated from the number of erythrocytes present as determined by the hemocytometer, allowing 40 per cent for the volume of the corpuscles in fresh blood. The results obtained by this method as given in Table V confirm those obtained by the previous method, showing that human erythrocytes are more sticky in acid solutions.

TABLE V.

Effect of pH on the Percentage of Erythrocytes Sticking to Glass.

Experiment A.		Experiment B.	
pH	Erythrocytes stuck on slide.	pH	Erythrocytes stuck on slide.
	<i>per cent</i>		<i>per cent</i>
4.1	100	5.5	79
5.2	92	6.5	62
5.6	80	8.0	13
8.0	20	8.8	5
10.0	29	9.7	11
10.1	2		

Experiment A.—Cells in 0.05 per cent serum. They were allowed to run under a cover-slip on a microscope slide 5 minutes after adding sodium hydroxide or hydrochloric acid. 5 minutes later the slide was inverted. After another 5 minutes the percentage sticking on the slide was determined by counting.

Experiment B.—Cells in 0.9 per cent sodium chloride, no serum. They were mounted on the slide immediately after mixing, and counts were not made until 15 minutes after inverting. In alkaline solutions many drop off during this longer interval unless they are allowed time to come to equilibrium after mixing and before mounting. Variations less than 10 per cent are not significant.

SUMMARY.

1. Washed erythrocytes, in both acid and alkaline solutions, hemolyze more rapidly when allowed to settle out on a clean microscope slide than when kept in suspension.

2. This is also true on slides coated with paraffin, paraffin oil, or vaseline, and on mica surfaces.

3. The presence of as little as 0.1 per cent serum inhibits such contact hemolysis, particularly in alkaline solutions.

4. Contact hemolysis is most marked on slightly soiled glass, and may occur so rapidly with unfixed cells in a hemocytometer that accurate counts are rendered impossible.

5. Erythrocytes are more sticky than normally in acid solutions and less sticky in alkaline solutions.

6. The increased stickiness of erythrocytes in acid solutions and their larger size during contact hemolysis in acid media provide some experimental evidence for the view that contact hemolysis is to be correlated with an attempt on the part of the corpuscles, or some ingredient of the corpuscle, to spread on the glass after the manner of leucocytes and invertebrate blood cells.

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THE TWORT-D'HÉRELLE PHENOMENON.

II. LYSIS AND MICROBIC VARIATION.

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PLATES 7 AND 8.

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In a preceding paper (1) the present knowledge of the phenomenon of transmissible microbic lysis discovered by Twort and by d'Hérelle was reviewed; there was described the method by means of which we dissociated a culture into two types of organisms, by merely allowing a culture of *Bacillus coli* to age. One, Type S, is very sensitive to destructive influences, such as desiccation and transmissible lysis. The other, on the contrary, is much more resistant. Besides these differences in vulnerability, the organisms are also distinguished by other properties; namely, motility and virulence.

Since that time we have had an opportunity to isolate a greater number of different types of *Bacillus coli*, all derived from the same original strain, as will be described in this article.

Characteristics of the Original Bacillus coli (coli O).

The culture employed was derived from a strain given by d'Hérelle to Bordet and Ciuca. Before beginning their experiment, Bordet and Ciuca made three successive isolations in order to start, as far as possible, with a culture arising from a single organism, and it was from this source that our culture was derived.

Besides the general properties of *Bacillus coli*, such as sugar fermentation and gas and indole production, this original strain of *Bacillus coli* possesses the following characteristics. The colonies on agar plates are large, flat, grape leaf-shaped, and very fluorescent. *Coli O* is non-motile and is very sensitive to the lytic agent, as is shown by the following tests.

Motility Test.—In order to test the motility of a strain, a stab culture is made into semisolid agar (0.5 per cent agar). If none of the organisms of a culture are motile, the growth is confined to the line of puncture. On the other hand, if they are all uniformly motile, they diffuse through the mass of agar and produce a uniform turbidity. If they have a variable motility the result is a combination of the two preceding pictures, that is to say, a dense growth appears close to the line of puncture and is surrounded by a diffuse zone of turbidity. This test is always controlled by a microscopic examination of broth cultures.

Resistance Test.—A suitable resistance test is found in the method of Bordet and Ciuca, slightly modified. These authors place a drop of lytic agent on a 3 hour culture of *B. coli* on agar slant. Resistant colonies grow on the path of clarification left by the drop. The number of these resistant colonies varies directly with the resistance of the strain.

In order to avoid the confusion that might result from the purely mechanical effect of the drop on the young growth, we prefer to place the drop on a sterile agar slant first, and then to seed the tube with *B. coli* 2 hours later. This method shows that *coli* O is very sensitive. Only very few or no resistant colonies appear in the zone of agar touched by the lytic agent.

Action of the Lytic Agent on coli O.—Bordet and Ciuca have shown that when a few drops of lytic agent are added to a broth culture of *coli* O, an almost complete dissolution occurs, with the exception of a very few resistant organisms. When transplanted on an agar slant these organisms produce a very scanty, irregular culture at the beginning, but after several passages the growth becomes more and more luxuriant and is distinguished from the original culture by certain characteristics. These organisms resist the lytic agent but have now acquired themselves lytic properties and become lysogenic, or capable of inducing dissolution in a culture of normal *Bacillus coli*. Moreover, when planted on slanted agar, a mucoid culture results. They are also less phagocytatable and more virulent.

We found that all of the bacilli comprising a culture of this modified *coli* do not possess all of these characteristics, which are rather shared by different types of organisms in the culture. The following experiment shows that the modified *coli* is a very heterogeneous culture.

Experiment 1.—0.5 cc. of increasing dilutions of lytic agent (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , straight broth) were mixed respectively with 0.5 cc. of a 12 hour broth culture of *coli* O diluted to 10^{-5} . Immediately afterward, these mixtures were plated in Petri dishes with 10 cc. of plain agar and incubated at 37°C . After 12 hours the result shown in Fig. 1 was obtained; Plate A is the control, *e. g.* a normal

culture of *coli* O, and the following plates, cultures of *coli* O mixed with an increasing quantity of lytic agent.

As shown in a preceding paper (1), the number of resistant colonies left varies in inverse proportion to the amount of lytic agent. But aside from this fact, it is possible to distinguish three types of colonies: (1) mucoid (Fig. 2); (2) non-mucoid and regular (Fig. 1, E and F); and (3) non-mucoid and irregular (Fig. 1, C and D).

Mucoid Colonies, or coli O R 1.

Such colonies are found only occasionally and it may be necessary to repeat the experiment several times to find one colony of this type. They are regular, large, convex, translucent, and non-fluorescent.

If a trace of one of these colonies is streaked on an agar plate, a curious picture results. The mucoid and translucent growth is studded with numerous opaque spots (Fig. 3, B). Among the isolated colonies some are entirely opaque, some entirely translucent, and some mixed. Transplanted, an opaque colony gives only a homogeneous opaque growth. On the contrary, a translucent colony reproduces the same mixture of translucent and opaque colonies. Thus, the translucent growth is a very unstable form which in an ordinary culture is very quickly overgrown by the more stable opaque form. In order to avoid complications, only the latter form will be considered under the distinctive denomination of *coli* O R 1.

Coli O R 1, like the original *coli* O from which it is derived, is non-motile but is completely resistant to the lytic agent. As is shown by the following test, it is not lysogenic or capable of inducing dissolution in a normal culture of sensitive *Bacillus coli*.

Lysogenic Test.—The technique is based on the fact that lysogenic organisms added, even in a very small number, to a young broth culture of sensitive *B. coli* produce lysis of this culture. The organisms to be tested are planted in 5 cc. of broth (pH 8.0). When the culture is 18 hours old, it is centrifuged and 0.5 cc. of the clear supernatant fluid, still containing a small number of organisms, is added to 5 cc. of a young broth culture (3 hours, pH 8.0) of normal *coli* O. When the growth of the mixture, compared with a normal culture of *coli* O as control, exhibits evidence of inhibition and dissolution, the test is positive.

This is not the case for *coli* O R 1.

To summarize, *coli* O R 1 is a non-motile, very resistant, and non-lysogenic organism producing large, round, mucoid, and non-fluorescent colonies.

Non-Mucoid Regular Colonies, or coli O R 2.

These colonies are found in the two last plates of Experiment 1; that is to say, in those plates in which the lytic agent is present in high concentration (Fig. 1, E and F). They are small, round, convex, hyaline, and non-fluorescent and retain these characteristics when transplanted on agar plates (Fig. 3, A). *Coli* O R 2 also is very resistant, non-lysogenic, and non-motile.

Non-Mucoid Irregular Colonies.

They exist only in the plates in which the lytic agent is present in moderate quantity (Fig. 1, Plates C and D). They are large, flat, non-fluorescent, and extremely irregular.

Experiment 2.—A trace of an irregular colony was streaked on an agar plate. In the first streaks the growth was scanty and irregular; but in the subsequent streaks the colonies became less irregular, showing sometimes only a slight indentation confined to a portion of the edge, and finally, perfectly regular large flat colonies could be found in the last streaks.

Irregular colonies can be transplanted in series many times with the same result. Nevertheless, after a certain number of generations, the proportion of regular colonies appearing in the last streaks increases.

Experiment 3.—An irregular colony, or the irregular edge of a partially irregular colony, was planted in broth. A very slowly growing culture resulted which was lysogenic.

Irregular colonies are thus lysogenic. On the other hand, the regular colonies found in the last streaks have lost this property, as is seen in the following experiment.

Experiment 4.—A regular colony, or the regular edge of a partially irregular colony, was streaked on an agar plate. Only regular colonies appeared. They were large, flat, and non-fluorescent. Transplanted in broth, they gave a normal rapidly growing culture of non-lysogenic and non-resistant organisms.

Thus it is evident that the lysogenic property is intimately related to this irregular growth first described by Bordet and Ciuca (2). This is not only the case for *Bacillus coli* but it is a general rule, since similar observations have already been made by Kuttner for typhoid bacilli (3), by Wollstein for Shiga bacilli (4), and by myself for staphylococci (5).

It is evident also that irregular colonies are diseased colonies and that indentations must be considered as lesions. Our experiments show that when the diseased material is streaked on agar plates, the lesions are very numerous in the first streaks but gradually disappear in the following ones and that in the last streaks only perfectly regular, healthy, and non-lysogenic colonies are found.

It results also from the preceding observations that all the organisms composing a culture of *coli* O do not respond in the same way to the lytic agent. (1) Some are so sensitive that they are dissolved even by very dilute filtrate (spots of clarification, Fig. 1, Plate B). (2) Some are just resistant enough to survive and grow in the presence of a moderate quantity of lytic agent, but they are still weak organisms, and, as they are more or less affected by the lytic agent, they produce diseased irregular colonies. They are the lysogenic organisms of Bordet and Ciuca. When streaked on agar plates, they can recover and produce in the last streaks regular, large, flat colonies made up of sensitive and non-lysogenic organisms. (3) Only a few individuals are sufficiently resistant to survive the strong action of concentrated lytic agent. They are absolutely invulnerable to the lysis and produce regular, small, convex, and hyaline colonies of very resistant and non-lysogenic organisms (*coli* O R 2). (4) Among these very resistant bacilli only a very few are mucoid.

The three principal characteristics of the modified *coli* of Bordet and Ciuca, *i.e.* resistance, lysogenic properties, and mucoid growth, are thus shared among different organisms.

$$B. coli O + \text{lytic agent} = \left\{ \begin{array}{l} \text{Irregular lysogenic} \\ \text{colonies} \\ \\ \text{Regular non-lysogenic} \\ \text{colonies} \end{array} \right\} \left\{ \begin{array}{l} \text{Coli O R 1} = \text{Resistant,} \\ \text{mucoid} \\ \\ \text{Coli O R 2} = \text{Resistant,} \\ \text{non-mucoid} \end{array} \right.$$

Action of the Lytic Agent on coli S and coli R.

As described in a preceding paper (1), two different types of organisms, *coli* S and *coli* R, have been obtained by allowing *coli* O to age. Both types have lost the property of producing mucoid growth when submitted to the lytic agent.

Experiment 5.—6 hour broth cultures of *coli* O and *coli* R were added respectively to 0.2 cc. of lytic agent, and incubated at 37°C. When a loopful of each partially dissolved culture was planted on agar slants 36 hours later, a scanty and irregular culture resulted which, after several passages, grew more readily, indeed, but never became mucoid.

This result is confirmed and explained by the following experiment.

Experiment 6.—The technique used in Experiment 1 for *coli* O was applied to *coli* S and *coli* R. Again irregular lysogenic colonies were found in the plates containing weak lytic agent, and regular resistant colonies in the plates containing concentrated lytic agent. But in any case, in spite of a large number of experiments, no mucoid colonies have been found.

In other words, neither *coli* S nor *coli* R contains any mucoid organism, or, at least, any organism capable of becoming mucoid.

Another striking observation can be made in this experiment. On account of its greater resistance, *coli* R gives a greater number of round resistant colonies and only a very few irregular lysogenic colonies. Moreover, when streaked on agar plates, the irregular growth cannot be preserved any length of time; hence, at the end of three or four passages, the growth is almost completely regular. Thus, *coli* R not only is less affected by the lytic agent than the more sensitive strains, *coli* O and *coli* S, but also recovers more quickly.

As the ultimate result of the lytic agent on *coli* S and *coli* R we thus obtain resistant, non-lysogenic, non-mucoid organisms. These organisms we shall term *coli* S R and *coli* R R respectively. *Coli* S R is non-motile, and, when transplanted, grows very slowly, producing only a slight turbidity in broth and very small round hyaline colonies on agar plates. *Coli* R R, on the other hand, is very motile and grows quickly.

$$\begin{array}{l}
 B. coli O + age = \left\{ \begin{array}{ll} Coli S + lytic agent = Resistant coli S R & (non-motile) \\ (non-motile) & (non-motile) \end{array} \right. \\
 \left\{ \begin{array}{ll} Coli R + lytic agent = Resistant coli R R & (motile) \\ (motile) & (motile) \end{array} \right.
 \end{array}$$

Further Variations of the Modified coli.

An agar slant of modified *coli*, obtained by Bordet and Ciuca, was kept in the ice box for several months before being used in the following experiments.

Experiment 7.—The old modified *coli* was transplanted each day on agar slants. The culture remained mucoid and lysogenic.

Experiment 8.—A trace of the modified *coli* was streaked on an agar plate and after 12 hours incubation two types of colonies were found: the more numerous were mucoid, the others—only a few—were non-mucoid. Both types of colonies were round and non-lysogenic.

Experiment 9.—Both types were streaked respectively on agar plates, and since this first isolation two pure cultures have been obtained which breed true—a mucoid one, or *coli* M 1 (Fig. 4, B), and a non-mucoid one, or *coli* M 2.

$$B. coli O + \text{lytic agent} = \text{Modified } B. coli + \text{age} = \begin{cases} coli M 1 \\ (\text{motile, mucoid}) \\ \\ coli M 2 \\ (\text{motile, non-mucoid}) \end{cases}$$

(non-motile)

As a matter of fact, it will be seen later that these strains cannot be identified with any of the types above described.

Characteristics and Evolution of coli M 2.

When streaked on agar plates, *coli* M 2 produces large, flat, pale blue, translucent, and non-fluorescent colonies. Different individuals of that type vary greatly in their motility, as observed by microscopic examination and by planting in semisolid medium. Submitted to the resistance test, they show an average resistance; *i.e.*, they do not possess the absolute invulnerability of *coli* R R or *coli* S R, but the path of clarification left by the drop on an agar slant of *coli* M 2 can be completely recovered by a resistant growth as early as 12 hours after seeding. The lysogenic test is negative. All of these characteristics are maintained indefinitely. When freshly isolated, *coli* M 2 is spontaneously agglutinable in plain broth. But this character is transient and disappears after a few passages in broth or on slanted agar.

The most interesting property of *coli* M 2 is its remarkable ability to give mucoid growth when again submitted to the lytic agent.

Experiment 10.—0.2 cc. of lytic agent was added to a broth culture of *coli* M 2 (6 hour, pH 8.0). A rather easy and almost complete dissolution occurred. After 24 hours incubation, a loopful of the partially dissolved culture was streaked on agar plates and gave the result shown in Fig. 4, A. In the first streak, in which the living bacilli and the lytic broth had been spread in abundance, the growth was scanty and irregular, but became less and less irregular in the following streaks, and was finally completely regular in the last streaks. This, of course, was to be expected from our former observations, but the remarkable fact was that a certain number of mucoid colonies was present in the first streak, very often covering the debris of an almost completely dissolved irregular colony of *coli* M 2 (Figs. 5 and 6).

Experiment 11.—Same experiment as the preceding one, but the sample of the dissolved culture was taken and streaked only after 3 days incubation. In this case almost all of the colonies were mucoid.

$$\begin{array}{lcl} B. coli M 2 + \text{lytic agent} & = & B. coli M 1 \\ (\text{non-mucoid}) & & (\text{mucoid}) \end{array}$$

It may be concluded that different types of *coli* differ greatly in their ability to give a mucoid growth when submitted to the action of the lytic agent. Some, like *coli* S and *coli* R, lack this property entirely; others, like *coli* O, have it to a certain extent; and some, like *coli* M 2, have it to a very high degree.

Characteristics and Evolution of coli M 1.

Streaked on agar plates, *coli* M 1 gives very large colonies, extremely mucoid, opaque, and fluorescent. Like *coli* M 2, these organisms have a variable motility, an average resistance, and no lysogenic properties.

Experiment 12.—*Coli* M 1 was streaked every day on agar plates, and retained these characteristics even after a great number of passages. But occasionally a colony showed on a portion of its edge a kind of indentation where the growth was non-mucoid. This transformation of mucoid into non-mucoid growth has a very typical aspect which can be seen in Fig. 7.

Experiment 13.—A trace of the non-mucoid material of the indentation described in the foregoing experiment was streaked on agar plates and gave a pure culture of non-mucoid and non-motile organisms (*coli* M 1 a) which possesses absolutely all the characteristics of *coli* O, and which, therefore, can be considered as a reversion to the original type.

With the exception of this occasional reversion, *coli* M 1 is very stable on agar plates, since only mucoid colonies have been observed

in the course of more than 50 platings. *Coli* M 1 is very stable also when kept growing in a synthetic medium, as the following experiment shows.

Experiment 14.—*Coli* M 1 was seeded in a tube of synthetic medium, composed as follows:

Water	1,000 cc.
Glycerol	30 cc.
Sodium chloride	5 gm.
Calcium chloride	0.1 gm.
Magnesium sulfate	0.2 gm.
Dipotassium phosphate	2.0 gm.
Ammonium lactate	12.0 gm.

pH 7.4

Transplantations were renewed daily. At the same time a loopful of each 24 hour culture was streaked on agar plates, in order to control the behavior of *coli* M 1 in the synthetic medium. Nothing but mucoid colonies was found in the plates in the course of more than fifteen passages of *coli* M 1 in synthetic medium.

Quite different was the result when *coli* M 1 was planted in plain broth.

Experiment 15.—Same experiment as the preceding one, but plain broth was substituted for synthetic medium. Since the first passage in plain broth a certain number of non-mucoid bacilli has appeared, as shown by the corresponding plate in which, among mucoid colonies, a certain number of non-mucoid colonies was found. At the end of ten passages in plain broth, almost all the bacilli were non-mucoid.

Plain broth realizes, then, a condition which does not exist in agar plates or in synthetic medium, and which induces the transformation of the mucoid *coli* M 1 into a non-mucoid form, or *coli* M 1 b.

This new non-mucoid form derived from *coli* M 1 in broth is not at all similar to the non-mucoid reversion, or *coli* M 1 a, which appears occasionally on agar plates. The first is extremely motile, while the latter is not. Nor is it similar to the motile non-mucoid modified *coli* described above under the denomination of *coli* M 2, because *coli* M 2, as we have seen, becomes very easily mucoid again when submitted to the lytic agent, while *coli* M 1 b never reverts to the

mucoïd form, even in the presence of lytic agent, as is shown in the following experiment.

Experiment 16.—5 drops of lytic agent were added respectively to 6 hour cultures of *coli* M 2 and *coli* M 1 b, and both mixtures were allowed to incubate at 37°C. for several days. A loopful of each mixture was streaked every day on agar plates in order to control the appearance of mucoïd bacilli. While numerous mucoïd bacilli have already appeared since the 1st day in the mixture of *coli* M 2 with lytic agent, not one could be detected in the mixture of *coli* M 1 b, even after 5 days of incubation.

$$\begin{array}{l}
 \left. \begin{array}{l} B. coli M 1 \\ (mucoïd, \\ motile) \end{array} \right\} \begin{array}{l} \text{In synthetic medium} = \text{Coli M 1} \\ \hspace{10em} (\text{mucoïd, motile}) \\ \text{On agar plates} = \left\{ \begin{array}{l} \text{Coli M 1} \\ \hspace{1em} (\text{mucoïd, motile}) \\ \text{Coli M 1 a} = \text{Coli O} \\ \hspace{1em} (\text{non-mucoïd, non-motile}) \end{array} \right. \\ \text{In plain broth} = \text{Coli M 1 b} \\ \hspace{10em} (\text{non-mucoïd, motile}) \end{array}
 \end{array}$$

A striking characteristic of *coli* M 1 b is that of giving an extraordinary granular growth when freshly isolated and transplanted in broth. Soon after transplantation, heavy clumps appear, which sediment at the bottom of the tube, leaving a clear supernatant broth.

This property led us to make a rather unexpected parallel with the recent observations of De Kruif on the bacillus of rabbit septicemia (6). This author observed the dissociation of the bacillus into two types: one is extremely virulent, produces opaque and very fluorescent colonies on agar plates, and grows diffusely in broth (Type D); the other type is avirulent, produces translucent and weakly fluorescent colonies, and gives granular growth in broth (Type G).

The similarity with our last findings is evident. Like Type D, *coli* M 1 gives opaque, very fluorescent colonies, and grows diffusely in broth; on the other hand, *coli* M 1 b, like Type G, gives translucent and weakly fluorescent colonies and produces granular growth in broth. According to recent experiments of De Kruif, Type D is able to change into Type G when kept growing in broth; *coli* M 1 b also appears in broth cultures of *coli* M 1, as we have seen. Type G never reverts to Type D; similarly no reversion from *coli* M 1 b to *coli* M 1 has thus far been observed, even in the presence of lytic agent. Because of this parallelism, it seems that the facts pointed out by

De Kruif are not a special feature of the bacillus of rabbit septicaemia, but have a more general bearing.

Besides, our attention has been called repeatedly to similar facts reported by Arkwright (7), who found that *Bacillus dysenteriae*, *Bacillus typhosus*, *Bacillus paratyphosus*, and *Bacillus enteritides*, could be made to yield two forms, the rough ("R") form, which makes stable emulsions, and the smooth ("S") form, which is spontaneously agglutinable. This distinction is similar to that, for instance, in the case of *coli* M 1 and *coli* M 1 b, and also to that in the case of *coli* M 1 and *coli* M 2.

General Properties of the Different Types.

In the course of our studies on the transmissible autolysis of *Bacillus coli*, we have thus isolated ten different types of organisms, all

TABLE I.

Type.	Resistance.	Motility.	Mucoid growth.	Ability to yield mucoid growth.	Fluorescence.	Seroagglutination.
O	+	0	0	+	++++	0
OR 1	++++	0	++++		0	
OR 2	++++	0	0	0	0	
S	+	0	0	0	++	+++
SR	++++	0	0	0	0	+++
R	++	++++	0	0	++	++++
RR	++++	++++	0	0	0	++++
M 1	+++	+++	++++		++++	++++
M 1 a	+	0	0	+	++++	0
M 1 b	++	++++	0	0	++	++++
M 2	+++	+++	0	++++	0	++++

derived from the original *Bacillus coli* of Bordet and Ciuca. They are perfectly distinguished from each other by striking characteristics, such as size, shape, opacity and fluorescence of colonies, mucoid or non-mucoid growth, ability to yield mucoid growth in the presence of lytic agent, motility, resistance to desiccation and to lytic agent, etc. (Table I). The specific properties of the colon bacillus are preserved; namely, the fermentation of carbohydrates, with the exception of saccharose, and the production of indole. The following experiment deals with the agglutination reaction.

Experiment 17.—Rabbits were immunized respectively with *coli* O, *coli* S, and *coli* R. The agglutinating power of the three antisera obtained was tested on nine of our strains by the following technique advised by Bordet and Ciuca. Increasing dilutions of the three antisera were made with plain broth and 1 cc. of each dilution of each serum was seeded respectively with one of the nine strains and incubated at 37°C. When the bacilli grew in clumps, the result was considered as positive. When the growth was diffuse the result was considered negative. The results are shown in Table II.

TABLE II.

Antiserum.	Titer of antiserum.	Strain.								
		O	S	S R	R	R R	M 1	M 1 a	M 1 b	M 2
O	1:10	—	++	+	++++	++++	++++	—	++++	++++
	1:100	—	+	—	++++	++++	++	—	++	—
	1:500	—	—	—	++	++	+	—	+	—
	1:1,000	—	—	—	—	—	—	—	—	—
	1:5,000	—	—	—	—	—	—	—	—	—
S	1:10	—	++++	++++	++	++	++++	—	++	++
	1:100	—	++++	++++	++	++	++++	—	++	++
	1:500	—	++	++	+	++	++++	—	++	++
	1:1,000	—	+	—	+	+	++++	—	+	++
	1:5,000	—	—	—	—	—	++++	—	+	++
R	1:10	+	++++	++++	++++	++++	++++	+	++++	++++
	1:100	+	++++	++++	++++	++++	++++	+	++++	++
	1:500	—	++++	++++	++++	++++	++++	—	++++	++
	1:1,000	—	++++	++	++++	++++	++++	—	++++	++
	1:5,000	—	++	—	++++	++++	++++	—	++	++

Two interesting facts appeared in this experiment. (1) While all the other strains grew in clumps in the presence of any of the three antisera, only the original *coli* (*coli* O) and the reversion to the original type (*coli* M 1 a) were not agglutinable, even by their corresponding antiserum, *i.e.* the serum obtained from a rabbit immunized with *coli* O, which, however, agglutinated the other types. It is very interesting that Type M 1 a, which we already considered as a reversion on account of its similarity with the original *Bacillus coli*, shows the same lack of agglutinability as *coli* O. (2) The titer of the anti-*coli* S serum and of anti-*coli* R serum was greater than 1:5,000, while,

on the other hand, the power of anti-O serum was much lower and hardly reached a titer of 1:500. *Coli* O is not only non-agglutinable but possesses also weak antigenic power.

DISCUSSION.

What is the nature of the different variations above described?

The notion of contamination can be disregarded with certainty, not only in consideration of the care constantly taken but because it has been possible to reproduce the experiments at any time, always with the same expected result, and because a certain number of our types have been isolated simultaneously in Brussels by Bordet and Ciuca from the same original strain of *Bacillus coli*.

The range of the variation never goes beyond the limit of the species: all of the strains still possess the specific properties of *Bacillus coli*.

All of the types, once isolated and regularly transplanted, keep their individuality even after several months. They are thus stable. Nevertheless, they are not all uniformly stable, and certain types, like *coli* M 1, for instance, still readily undergo changes under certain definite influences.

It is out of the question also to say that the eleven types coexisted in the original strain, because in order to admit such a coexistence it would be necessary to imagine that at least eleven different organisms, each of them representing one of our variations, came together through the three successive isolations performed before the present studies were begun. We have thus been forced to accept the conclusion that the different types observed are the result of changes occurring in the original *Bacillus coli* in the course of these studies. The new types always appear under certain definite conditions of the environment, but it is still impossible to determine whether the external influence is the direct cause of the variation or only an occasional factor which makes apparent, by mere selection, a modified germ already present but in too small numbers to be detected, and produced independently of the environment itself.

It is a common observation that transmissible lysis of a bacterium, induced under certain influences such as stool filtrate, peritoneal exudate, tissue extracts, and vaccine, happens in a rather haphazard

and irregular fashion and seems a question of luck. This fact is easily explained by the hypothesis of a bacteriophage virus. But if the phenomenon is not due to a virus, but as suggested by Bordet and Ciuca, to some autolytic vitiation of the bacteria, the irregularity with which the phenomenon is promoted must find its cause in the bacteria themselves. It is possible that a given bacterium is not always capable of starting the lysis. Various forms of *Bacillus coli* appear to differ in their properties, for example in their behavior toward the lytic agent. Certain forms become mucoid in the presence of the lytic agent. Similarly, it may be possible for only a certain form to undergo autolysis. In order to start the dissolution it is necessary to employ the proper forms of bacteria.

SUMMARY.

1. When the few individuals still alive in a dissolved culture of *Bacillus coli* are transplanted on slanted agar, a culture results which possesses new characteristics. First observed by Bordet and Ciuca, this culture received the temporary name of modified *coli*.

In the study described above, we found that this modified *coli* is very heterogeneous and that its three principal characteristics, resistance to lysis, lysogenic properties, and mucoid growth, are shared among different types of organisms that can be isolated when the normal original *coli* (*coli* O) is plated together with increasing quantities of the lytic agent: (a) a certain number of bacilli are just resistant enough to survive and grow in the presence of a moderate quantity of lytic agent, but they are still more or less sensitive and produce diseased, irregular, and lysogenic colonies; (b) a few of the organisms are able to resist concentrated lytic agent; they are entirely resistant and give round, healthy, and non-lysogenic colonies (*coli* O R 2); and (c) among these resistant bacilli only a very few are mucoid (*coli* O R 1). All these types are not motile and not fluorescent.

2. The original *coli*, when allowed to age, can be dissociated, as we have shown in a preceding paper (1), into two types of organisms, the non-motile *coli* S and the very motile *coli* R. Submitted to lysis, *coli* S gives a very small number, *coli* R a much greater number of resistant organisms (*coli* S R and *coli* R R), but both types never yield any mucoid growth.

3. An old culture of the modified *coli* obtained by Bordet and Ciuca, when streaked on agar plate, gives two types of colonies: a mucoid and fluorescent type (*coli* M 1) and a non-mucoid and translucent type (*coli* M 2). Both types are motile.

Coli M 2, once isolated, keeps its individuality even after several passages in artificial media, but if again submitted to the lytic agent, a great number of mucoid bacilli are found among the organisms which are still alive.

Consequently, different types of *Bacillus coli* differ greatly in their ability to give a mucoid growth when submitted to the lytic agent. Some, like *coli* S and *coli* R, do not possess this property at all. Others, like *coli* O, possess it to a certain extent, and some, like *coli* M 2, have it to a very high degree.

4. The mucoid and motile *Bacillus coli* M 1, when streaked every day on agar plates, remains indefinitely mucoid and motile, but occasionally a mucoid colony shows an indentation made up of non-mucoid growth, which, transplanted, gives a pure culture of non-mucoid and non-motile organisms, *coli* M 1 a. This new type possesses all the characteristics of the original strain of *Bacillus coli*, and therefore must be considered as a reversion.

5. The mucoid and motile *Bacillus coli* M 1, kept growing in synthetic medium, remains perfectly stable; on the other hand, when it is transplanted in broth, *Bacillus coli* M 1 turns very quickly into a non-mucoid but still very motile organism, or *Bacillus coli* M 1 b. This last type, which produces translucent colonies on agar and grows granular in broth, never reverts to the mucoid form, even in the presence of lytic agent.

6. A single strain of *Bacillus coli* has thus been made to yield eleven different forms, all distinguished by striking characteristics, but still possessing the specific properties of *Bacillus coli*.

Nine of these forms have been submitted to antisera prepared with three different types (*Bacillus coli* O, *Bacillus coli* S, and *Bacillus coli* R). While seven out of these nine strains were agglutinated by any of the three antisera, only the original *Bacillus coli* (*Bacillus coli* O) and the reversion to the original type (*Bacillus coli* M 1 a) were not agglutinable, even by their corresponding antiserum; *i.e.*, the serum obtained from a rabbit immunized with *Bacillus coli* O, which, however, agglutinated the other types.

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EXPLANATION OF PLATES.

PLATE 7.

FIG. 1. Experiment 1. *Coli* O plated with increasing quantities of lytic agent ($O, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}$). Plate A. Control; normal culture of *coli* O. Plates B, C, and D. *Coli* O + moderate quantities of lytic agent; note the great number of irregular colonies. Plates E and F. *Coli* O + concentrated lytic agent; note the presence of only small regular colonies. No mucoid colonies are found in this experiment.

PLATE 8.

FIG. 2. When Experiment 1 is repeated several times, a mucoid colony can occasionally be found. Here is a plate of *coli* O mixed with concentrated lytic agent. Besides the small regular colonies, two large mucoid colonies are visible.

FIG. 3. Plate A. A small round colony of Plate F, Fig. 1, was planted and gave a pure culture of *coli* O R 2. Plate B. The lower mucoid colony seen in Fig. 2 was transplanted and gave a mucoid culture of *coli* O R 1; note the translucent growth studded with opaque spots.

FIG. 4. Plate A. Experiment 10. A loopful of a partially dissolved culture of *coli* M 2 was streaked on an agar plate. In the first streak the growth is very irregular and contains a certain number of mucoid colonies. In the second, the colonies have already become less irregular, and in the third they are normal. Plate B. Mucoid growth of *coli* M 1.

FIG. 5. Magnification of a portion of the first streak of Plate A, Fig. 4. Showing mucoid colonies among the very irregular colonies of *coli* M 2.

FIG. 6. The same. A mucoid colony recovering the debris of a colony of *coli* M 2.

FIG. 7. Magnification of the edge of a colony of *coli* M 1 where an indentation of non-mucoid growth (light) issues from the mucoid growth (dark).

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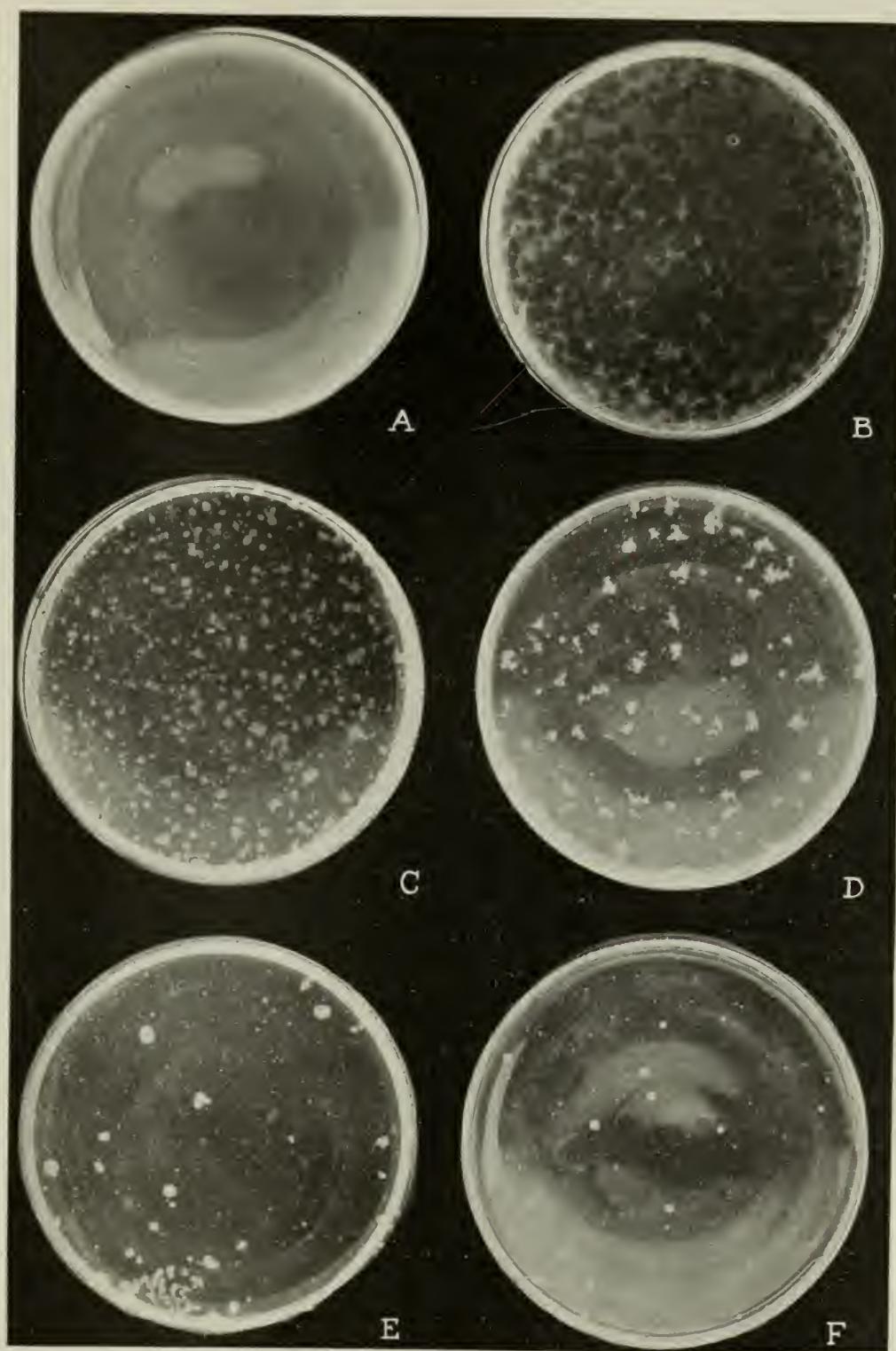


FIG. 1.

(Gratia: Twort-d'Hérelle phenomenon. II.)

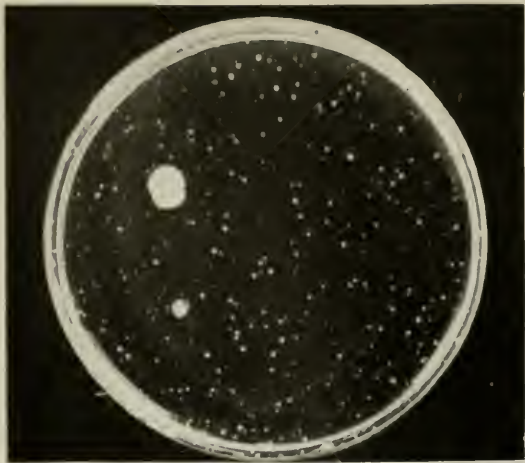


FIG. 2.



FIG. 3.

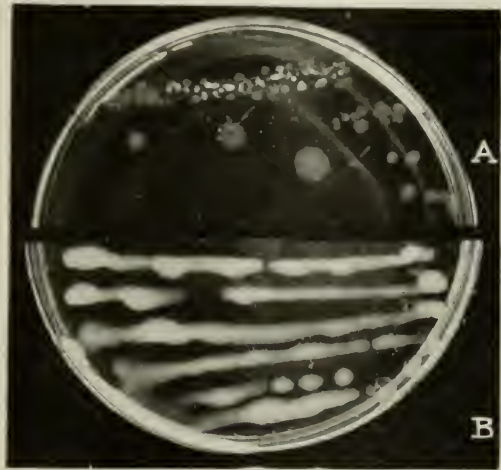


FIG. 4.



FIG. 5.



FIG. 6.

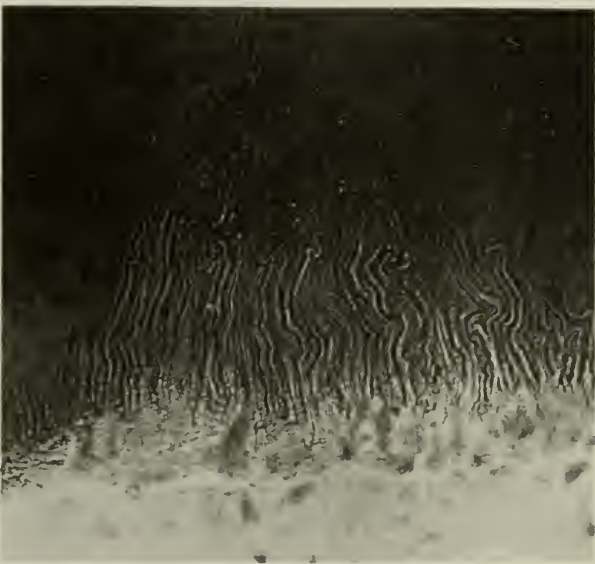


FIG. 7.

(Gratia: Twort-d'Hérelle phenomenon. II.)

THE CULTIVATION AND BIOLOGICAL CHARACTERISTICS OF SPIROCHÆTA OBERMEIERI (RECURRENTIS).

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The spirochetes of relapsing fever were first cultivated by Noguchi¹ in 1912. He employed a culture medium consisting of ascitic fluid to which a fragment of fresh sterile rabbit kidney had been added. Noguchi placed considerable stress on the character of the ascitic fluid used, selecting only such as gave fibrin formation throughout the medium after inoculation. The culture fluid was inoculated with infected rat blood, and covered with a layer of paraffin oil. By this method he succeeded in growing these organisms through a number of generations. The maximum growth occurred between the 4th and 6th days, at which period subcultures were made. This technique was successfully applied by Plotz² in cultivating these spirochetes from the blood of patients. Subsequent to Noguchi's report, Hata³ cultivated *Spirochæta obermeieri* in horse serum diluted with saline solution and coagulated at 70°C. He found that in the presence of kidney the organisms lived only a short time, but that on substituting a fragment of blood clot for kidney, they survived and remained infective for a long period. Recently Ungermann⁴ has reported successful results with inactivated rabbit serum without kidney.

The principle underlying the cultivation of *Spirochæta* was discovered by Noguchi. However, there are certain details in the preparation of the medium and the conditions controlling the growth of these organisms which had not been fully studied.

In our attempts to cultivate the spirochetes of relapsing fever by the different methods outlined above, we found the Noguchi method the most satisfactory, but in our hands the results were inconstant. Occasionally a good initial growth was obtained, but it was not always possible to carry the culture to a second generation. Fre-

¹ Noguchi, H., *J. Exp. Med.*, 1912, xvi, 199.

² Plotz, H., *J. Exp. Med.*, 1917, xxvi, 37.

³ Hata, S., *Centr. Bakt., 1te Abt., Orig.*, 1914, lxxii, 107.

⁴ Ungermann, E., *Arb. k. Gsndhtsamte*, 1919, li, 114.

quently in the same medium, either no growth occurred in spite of a rich inoculum, or an abundant growth was suddenly terminated by rapid degeneration at the end of the 3rd or 4th day. It was evident that we were dealing with certain unknown variables in the culture media which in one instance made it suitable and in another unsuitable for the growth of the spirochetes. Furthermore, it was evident that even in a favorable medium changes were rapidly taking place which rendered it unfavorable. Therefore, it seemed desirable to analyze more fully the factors in the growth requirements of these organisms. *Spirochæta obermeieri* was the type used in these experiments.

Culture Media.

Constituents of the Media.—Our findings, with regard to the basic constituents of the most favorable culture media, agreed in the main with those of former workers, and may be summarized briefly as follows:

Animal fluids are necessary for the growth of *Spirochæta obermeieri*. Ascitic fluid, horse and rabbit serum serve this purpose equally well. Inactivation of the serum was found to be unnecessary, and coagulation undesirable. Dilution of the serum with one to two parts of normal salt solution does not impair its nutrient properties. Growth is more rapid in undiluted serum, but the viability of the culture is greater in diluted serum. The addition of kidney is not necessary.

The physical condition of the medium seems to exert an important influence. The growth activity of the culture is markedly increased by the presence of a small amount of agar or fibrin, as suggested by Noguchi, who found them favorable for *Leptospira icteroides*. Under these conditions, growth is more abundant and the spirochetes are found chiefly enmeshed in the agar or fibrin.

Reaction of the Media.—Recent investigations have shown that the concentration of hydrogen ions in the medium is an important limiting factor in affecting the growth and viability of microorganisms. Consequently a series of experiments was made to determine the effect of pH on the growth of the spirochetes. Media made up of ascitic fluid, horse and rabbit serum respectively were adjusted to various reactions, inoculated with the same amounts of infected

rat blood, covered with oil, and incubated. After 3 to 5 days the cultures were examined under the dark-field microscope and the reaction of the media was again tested. The following protocol is typical.

Horse and rabbit sera, to which 0.1 per cent agar had been added, were adjusted to different reactions by the addition of N NaOH or N HCl. 3 cc. of medium were put into a tube. All the tubes were inoculated with the same amount of infected rat blood and incubated at 30°C. for 3 days. The data are presented in Table I.

TABLE I.
Effect of the Reaction on Growth.

Tube No.	Rabbit serum.			Horse serum.		
	Initial pH.	Final pH.	Results.	Initial pH.	Final pH.	Results.
1	7.9	8.2+	0 Spirochetes dead.	7.9	8.2+	0 Spirochetes dead.
2	7.6	8.1	± 1 to 10 fields; degenerated.	7.6	8.1	± 1 to 10 fields; degenerated.
3	7.3	7.6	++ 1 or more to a field; active; in chains.	7.2	7.5	+++ 2 to 3 in a field; active; in chains.
4	6.9	7.1	+ 1 to 3 fields; active.	6.9	7.2	+++ 2 to 3 in a field; active.
5				6.4	6.6	0 None found.

These tests established two important facts: (1) that the reaction of the medium has a decided influence on the growth of the spirochete; and (2) that during the incubation, the reaction of the culture fluids changes progressively toward alkalinity irrespective of the initial reaction.

In media having a reaction below pH 7.0 or above pH 8.0 there was apparently no growth, while the maximum rate of multiplication occurred between pH 7.2 and 7.4. Even in an actively

growing culture rapid degeneration set in as soon as the reaction approached the limiting pH. This has been verified by repeated tests of the reaction of cultures which had suddenly died.

The progressive increase in the alkalinity of the media (Table I) is a factor of considerable importance in the cultivation of these organisms. This change occurred irrespective of the initial reaction of the medium or of the growth of the spirochetes, and was characteristic of all media made up largely of animal fluids. It was found that ascitic fluid, horse, sheep, and rabbit sera become progressively more alkaline after withdrawal from the body and exposure to the air. Felton, Hussey, and Bayne-Jones⁵ observed that spinal fluid changed from a pH 7.7 or 7.8 to 8.6 within a few hours. Ascitic fluid is also prone to marked rapid change, while animal sera change more slowly. The following experiment is illustrative.

Horse and rabbit sera were diluted with saline solution in the proportion of 1:3, the reactions were adjusted, and the fluid was distributed in 3 cc. amounts into a series of tubes. Tubes of ascitic fluid were also made. Another similar set of tubes was set up with serum and ascitic fluid which had been heated for 10 minutes in a water bath at 100°C. The results, after incubation for 2½ days, are given in Table II.

TABLE II.
Reaction Changes in Animal Fluids at Room Temperature.

Incubation time 2½ days.

Fluid.	Unheated.		Heated.	
	Initial pH.	Final pH.	Initial pH.	Final pH.
Horse serum (1 : 3).....	7.0	7.8	7.0	7.8
Rabbit " (1 : 3).....	7.2	7.7	7.2	7.8
Ascitic fluid.....	7.2	8.2+	7.2	8.2+
" " (1 : 1).....	7.3	8.2+	7.3	8.2+

The instability of the reaction is most likely due to a progressive loss of CO₂. This is indicated by the facts that the change is always toward alkalinity, that agitation hastens the change, and, finally, that heating to destroy enzymes does not prevent or even retard the

⁵ Felton, L. D., Hussey, R. G., and Bayne-Jones, S., *Arch. Int. Med.*, 1917, xix, 1085.

reversion. The loss of CO_2 would readily account for the increase in alkalinity. The relationship of CO_2 concentration in the blood and blood reaction has been fully discussed by L. J. Henderson.⁶

Stabilization of the Media.—It was evident that the next step was to find some means of stabilizing the culture fluids. Several methods suggested themselves, each of which was tested in turn.

1. The first procedure was to acidify the medium, agitate vigorously, and then adjust to the desired reaction. This led to the precipitation of the serum proteins which were not completely redissolved. The turbid fluid was not satisfactory for culture purposes.

2. We then determined the extent to which kidney tissue, as used by Noguchi, tends to regulate the reaction. It is well known that kidney tissue autolyzes with the liberation of acid, but the degree and rate of autolysis and diffusion of the acid were unknown. It was found that the kidney tissue gradually neutralizes the alkalinity of the medium but that diffusion is slow and the effect is not uniform on account of the number of variables that enter. As a result of the slow diffusion of the acid, the portion of the medium above the kidney may be decidedly acid, the upper portion still alkaline, while in between is a zone of graded reactions. The rate and degree of acidification depend roughly on the relation of the size of the kidney tissue to the amount of fluid used, as well as on the character and initial reaction of the fluid. With a piece of kidney of appropriate size and a suitable amount of animal fluid of a given reaction, the final reaction of the medium may be brought to about the desired level. For example, a fragment of kidney tissue, the size of a pea, added to 6 cc. of ascitic fluid of a reaction of pH 8.4 brought the reaction down to a pH of 7.2 in 2 days; a larger piece of kidney with the same amount of this ascitic fluid made the medium decidedly acid. But one could not predict in any given case what the final reaction would be. Noguchi's results with this method were presumably due to a proper proportion between ascitic fluid, reaction, and kidney tissue, and the fact that he made transplants before the reaction of the media had changed beyond the limits of growth.

3. Mineral oil as originally used by Noguchi was also found to retard the change in reaction. The higher the column of oil the

⁶ Henderson, L. J., *J. Biol. Chem.*, 1909-10, vii, 29.

less rapid the change. However, as shown in Table IV, the oil itself is not sufficient to prevent rapid alkalization of the medium to a point inhibitive to the growth of the spirochete.

4. Since the increase in alkalinity is presumably due to diffusion of CO₂, it seemed likely that a simple solution to the problem would be growing the organism in an atmosphere containing an appropriate amount of CO₂. The stabilizing effect of CO₂ is illustrated by the following experiment.

TABLE III.
Effect of CO₂ on the Reaction.
Changes of Media; Initial Reaction pH 7.3.

Incubation time 3 days. All tubes kept in air-tight jar under the same conditions.

Media.	Dextrose broth cultures of <i>B. coli</i> .	3 vol. per cent CO ₂ .	5 vol. per cent CO ₂ .
Ascitic fluid.....	7.7	7.4	7.3
“ “	7.7	7.5	7.3
Serum (1 : 2).....	7.3	7.3	7.3
“ “	7.3	7.3	7.3
Ascitic fluid + broth.....	7.5	7.3	7.3
“ “ + phosphate.....	7.3	7.3	7.3
Serum (1 : 2) + broth.....	7.3	7.3	7.3
Control without <i>coli</i> or CO ₂ .			
Ascitic fluid.....	8.0+		
“ “ + 1 per cent peptone.....	7.6		
“ “ + 1 “ “ egg albumin.....	7.8		

Test-tubes containing various kinds of culture fluids were placed in glass-topped jars. Into some of the jars were put dextrose broth cultures of *B. coli*, while to others different volumes per cent of CO₂ gas were added. The results are shown in Table III.

Similar experiments to test the ability of cultures to grow under these conditions gave satisfactory results. A simple procedure was to adjust the reaction of the ascitic fluid to pH 7.2, inoculate with infected blood, cover with about 1.0 cm. of oil, and put in jars together with two flasks of dextrose broth inoculated

with *B. coli*. Good growth was obtained in 4 to 5 days. The only objection to this method is that it requires resetting of jars every time a culture is examined.

5. Finally, the reaction of the medium was stabilized by the addition of buffer substances. Peptone, egg albumin, and phosphates were used. All had the desired effect, to a greater or lesser extent (Table IV), but phosphates in amounts greater than 0.2 per cent were somewhat toxic to the spirochete, while egg albumin was too weak a buffer. Peptone apparently met all the requirements.

TABLE IV.

Effect of Buffer and of Various Amounts of Oil on the Reaction.

Effect of buffer. Initial pH 7.2; time 5 days.		Effect of oil. Initial pH 7.4; time 2 days.	
Media.	Final pH.	Media.	Final pH.
Ascitic fluid.....	8.2+	Ascitic fluid.	9.0
“ “ + 2 cm. of oil.....	8.0	“ “ + 1 cm. of oil.	7.9
“ “ + 1 per cent pep- tone.....	7.8	“ “ + 2 “ “ “	7.9
Ascitic fluid + 1 per cent egg albumin.....	8.2	“ “ + 3 “ “ “	7.8
Ascitic fluid + 0.1 per cent phos- phate.....	8.0	“ “ + 4 “ “ “	7.7

Limiting Hydrogen Ion Concentration.

In the early experiments it was indicated that the reaction limits for the growth of *Spirochæta obermeieri* were pH 7.0 and 8.0 with an optimum at pH 7.2 to 7.4. On account of the shifting reaction during incubation, it was not possible to determine these limits accurately. Repeated tests with buffered media have given practically the same results. No growth was obtained in media having a pH value of 6.8 on the one hand, or 8.4 on the other. Growth takes place in the zone between pH 7.0 and 8.2, but is always most active at pH 7.3.

The following is a typical experiment.

Ascitic fluid containing 2 per cent peptone broth was divided into various lots, each adjusted to a different pH, and the media were distributed into tubes. Each tube was inoculated with the same amount of infected blood. After incubating 4 days at 30°C. the observations in Table V were made.

TABLE V.

Limiting Reactions of Growth of Spirochæta obermeieri.

Medium: Ascitic fluid + 2 per cent peptone broth. Incubation time 4 days.

Initial pH.	Final pH.	Results.
6.8	6.9	± An occasional live organism; many dead.
7.0	7.1	++ Active; chains of 2; 1 in 5 or 6 fields.
7.0	7.3	+++ Many active; chains of 2 and 3 elements; 1 to 2 per field.
7.4	7.5	++ Many active; 1 in 2 or 3 fields.
7.6	7.7	++ Active; chains of 2; 1 in 5 or 6 fields.
7.8	7.9	+ Fewer active; some degenerated.
8.0	8.1	+ About the same as 7.8.
8.2	8.3	± Many degenerated; occasional active spirochete.

Oxygen Requirements.

Some uncertainty exists regarding the oxygen requirements of these spirochetes. Noguchi⁷ recognizes their essential need of oxygen and calls them aerotropic anaerobes. Ungermann,⁴ on the other hand, considers them strict anaerobes. The idea that a

⁷ Noguchi, H., *The Harvey Lectures*, 1915-16, xi, 236.

layer of paraffin oil serves as an oxygen barrier is entirely wrong. Our early observations indicated that they were strictly aerobic, and the following experiments show this to be the case.

(a) *Oxygen Tension*.—Cultures of *Spirochæta obermeieri* were prepared and placed in a series of Noguchi jars. These jars were then exhausted and refilled with definite proportions of O and N gas, measured by means of a vacuum gauge, to give varying oxygen tensions. The jars were left at room temperature (27—30°C.) for 4 days, after which the cultures were examined.

A typical protocol follows.

Oxygen, vol. per cent }	40	20	18	14	9	5
Growth.	0	++	+	0	0	0
	A few surviving organisms; degeneration.	Good growth. 1 to 2 to a field; few degenerated.	1 to 5 or 6 fields; many degenerated.	Few surviving; marked degeneration.	Few surviving; marked degeneration.	Few living.

The best growth was obtained under normal oxygen tension. A decrease to 0.9 per cent atmospheric tension retards growth, while a greater decrease or increase in the amount of oxygen apparently prevents growth entirely.

(b) *Oil Layer*.—Since a layer of oil had been found of value in stabilizing the reaction of the medium, it was important to determine the maximum column that might be used without preventing adequate aeration. It was found, contrary to Ungermann's assumption, that oil does not exclude air, but apparently retards the diffusion of gases. In a series of cultures made with increasing columns of oil, most abundant growth was obtained in those tubes having a layer 0.8 to 1.5 cm. high. Presumably a column of oil greater than that is sufficient to prevent the normal ingress of air.

(c) *Column of Media*.—The height of the column of medium was found to be of comparatively little importance. This was to be expected since growth is always most abundant in the upper, well aerated portion of the column. However, it was found that, other conditions being the same, growth was better when the column of the medium did not exceed 4 to 5 cm. in height.

Method of Cultivation.

The method finally adopted for the cultivation of *Spirochæta obermeieri* is as follows:

TABLE VI.
Detailed History of Some Cultures and Transplants.
1. Horse Serum without Buffer.

Medium.*	Date inoculated.	Interval before examination.					
		4 days.	7 days.	10 days.	14 days.	21 days.	39 days.
Horse serum + 0.1 per cent agar.	Sept. 22	+ Few active.	++ Active forms free and in agar; many dead.	+++ Large numbers in agar; few free swimming; many dead.	++ Many live; more dead.	+ Few active; many dead in agar.	0 Culture dead.

2. Horse Serum with Buffer (Broth).

Medium.*	Date inoculated.	Interval before examination.						
		4 days.	7 days.	10 days.	14 days.	21 days.	39 days.	51 days.
Horse serum + 0.5 per cent broth + 0.1 per cent agar.	Sept. 22	+++ Numerous live organisms in agar; some free swimming.	+++ Many active in agar and free; some dead.	+++ Mostly in agar; many dead.	++ Many live spirochetes, but increasing number dead.	+	± Some active forms left.	0 Culture dead.

3. Horse Serum with Buffer (Peptone).

Medium.*	Date inoculated.	Interval before examination.			
		5 days.	10 days.	17 days.	23 days.
Horse serum + 1 per cent peptone + fibrin.†	Sept. 24	++ Many active; few dead.	++ Chains; active; chains in 1 colony.	+ Few active.	0 Contaminated.

4. *Horse Serum Diluted, with Buffer (Broth).*

Medium.*	Date inoculated.	Interval before examination.				
		5 days.	10 days.	23 days.	37 days.	49 days.
Horse serum + normal salt solution 1:1 + 0.5 per cent broth + fibrin.	Sept. 24	++ Many active; degenerated forms but none dead.	++ Active; chains; few degenerated.	++ Active; chains; many dead.	++ Active; colony.	++ Masses of active organisms in fibrin.
						0 Contaminated.

5. *Horse Serum Diluted, with Buffer (Egg Albumin).*

Medium.*	Date inoculated.	Interval before examination.						
		5 days.	10 days.	17 days.	23 days.	37 days.	49 days.	61 days.
Horse serum + normal salt solution 1:1 + 1 per cent egg albumin + fibrin.	Sept. 24	++ Active; no dead forms seen.	+++ Chains of 2 and 4; masses of organisms.	++ Only occasionally free; mostly in agar; many dead.	++ Some active; many dead.	++ Few active; many dead.	+++ Freeswimming; many active in fibrin. (Culture used for transfer and reaction test.)	
Horse serum + normal salt solution 1:2 + 1 per cent egg albumin + fibrin.	Sept. 24	++ Few live, active; none dead.	++ Few active; chains.	+++ Many active colonies in fibrin.	++ Some active; many dead.	++ Few active; many dead.	++ Few active; contaminated.	0

* Reaction adjusted to pH 7.2.

† The broth added contained 10 per cent peptone, so that the amount of peptone in the culture fluid was 0.5 to 1.0 per cent according to the amount of broth added.

‡ 1 drop of fresh rabbit blood. All tubes covered with 1 cm. of oil and grown at room temperature after the first 3 days.

The basic media consist of horse serum or rabbit serum diluted with one or two parts of saline solution, or of ascitic fluid undiluted. To the fluid used 1.0 per cent peptone broth is added (1.0 cc. of a 10 per cent peptone broth to 10 cc. of fluid), and the reaction adjusted to pH 7.2. The media are distributed in 3 to 4 cc. amounts into tubes approximately 1.0 cm. in diameter. The tubes are then inoculated with 1 drop of infected blood, or, if subcultures are being made, with 0.1 cc. of culture, and covered with a layer of oil about 1.5 cm. high. In initial cultures the rat blood furnishes the fibrin; in subcultures the fibrin is supplied by a drop of fresh rabbit blood added prior to the addition of the oil. By rotating the tube gently to obtain uniform distribution of the blood, a delicate fibrin basket forms which remains suspended from the oil. In place of fibrin 0.05 to 0.1 per cent agar may be used. The tubes are then incubated at a temperature of 28–30°C.⁸ After the culture has been well started, growth proceeds satisfactorily at room temperature.

Viability of Spirochæta obermeieri.

With the procedure described above, it was possible (1) to cultivate these spirochetes consistently from mouse or rat blood; (2) to maintain the cultures for periods of 3 to 7 weeks; and (3) to carry them on in successive subcultures. In undiluted serum the initial growth is more rapid, but degeneration sets in relatively early (about 2 weeks). In diluted serum or in ascitic fluid, growth is slower, but it extends over a much longer period (7 weeks). Successful subcultures have been obtained from cultures 17, 24, 30, 31, and 49 days respectively after inoculation. As a general routine, the first transplants were made after 12 to 15 days incubation, while in later cultures the interval was 20 to 30 days. A detailed history of some of the cultures is given in Table VI.

SUMMARY.

A study of the growth requirements of *Spirochæta obermeieri* resulted in the perfection of a method which enabled us (1) to culti-

⁸ The spirochetes grow well at 37°C., but degeneration changes tend to set in early.

vate the organisms consistently from the blood of infected mice and rats, (2) to maintain the viability of cultures for periods of at least 3 to 7 weeks, and (3) to carry them on in successive subcultures by transplanting at intervals of 2 to 4 weeks. This method is essentially the same as the Noguchi technique for the cultivation of the *Leptospira* group, but emphasizes control of the physicochemical factors that act to limit and prevent growth and prescribes the conditions necessary to counteract the injurious influences. The main facts may be briefly summarized as follows:

(a) Ascitic fluid, horse or rabbit serum may be used as culture fluids.

(b) These fluids become progressively more alkaline on exposure to air.

(c) Uniformly successful results depend chiefly on the proper adjustment and stabilization of the reaction.

(d) A balanced reaction can be secured by adding 1.0 per cent peptone broth or egg albumin as buffer, and covering the culture with a layer of oil.

(e) The reaction limits for growth and survival are between pH 6.8 and 8.2, with the optimum at pH 7.2 to 7.4.

(f) *Spirochæta obermeieri* is a strict aerobe, consequently in order to permit adequate aeration, the oil layer should not exceed 1.5 cm. in height.

THE IMPORTANCE OF DEXTROSE IN THE MEDIUM OF TISSUE CULTURES.

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Since the metabolism of sugar plays such an important part in sustaining animal life, it seemed of moment to ascertain what influence this substance might have upon cells of tissue cultures which, although living necessarily under more or less anomalous conditions, nevertheless furnish the most satisfactory method known at present for the study of the behavior of cells when exposed directly to any given substance.

Material and Method.

For this investigation over 500 cultures of the connective tissue of chick embryos (7 to 9 days incubation) were prepared in media from which dextrose had been omitted or which contained from 0.25 to 5 per cent of this substance. Various kinds of media were used: white of egg, amniotic or allantoic fluid from chick embryos after 12 or 14 days incubation, Locke's solution containing 10 to 40 per cent bouillon,¹ Locke's solution with from 0.1 to 1 per cent peptone, Locke's solution containing gelatin, Locke's solution with white of egg, and Locke's solution alone.

The cultures were neither bathed nor retransplanted, as the object was to note the effect produced by a given medium without renewing it.

To facilitate the recognition of any abnormal structures that might arise in the cells as a result of other factors in the medium, it was necessary to determine the structure of the normal connective tissue

¹ 85 cc. of NaCl 0.9 per cent plus KCl 0.042 per cent plus CaCl₂ 0.025 per cent plus NaHCO₃ 0.02 per cent plus 15 cc. of chicken bouillon plus dextrose 0.5 per cent is known as Locke-Lewis solution.

cell. For this, sections of chick embryos and films of fresh embryonic tissue were used.

Normal Cells.—The connective tissue cell of the young chick embryo was found to be large, irregular, and often spindle-shaped, with a large round or oval nucleus. In some cells the nucleus was bent, in others it was double. The cytoplasm was homogeneous except for numerous rod- and filament-shaped mitochondria, an occasional fat droplet, and in some instances a few minute neutral red bodies. The position of the centrosome was frequently indicated by a clear region near the nucleus. The processes were elongated and usually adherent to those of neighboring cells or to the cover-glass. The cells had the same characteristics in the sections and in the films, provided the films had been prepared in Locke-Lewis solution containing dextrose and were not kept under observation for more than a few minutes. These cells corresponded substantially to those described from sections of the embryo by Duesberg (1910), and in tissue cultures by Lewis and Lewis (1915), Levi (1916), and W. H. Lewis (1919). They did not contain either vacuoles or specific granules.

Abnormal Cells.—When these cells were placed in certain media, other structures made their appearance, some within a few minutes, others not for many days. These abnormalities were for the most part of two kinds: a clear, slightly refractive vacuole and a more or less opaque, rather large granule. In addition to these, there were sometimes giant centrospheres, blebs, liquefaction of the homogeneous cytoplasm, and an accumulation of fat in the cells.

The vacuoles were encountered much more frequently than were the granules. Although never seen in well fixed sections of embryonic tissue, they did form in the cells in film preparations, especially in normal salt solution or Locke's solution lacking dextrose. Prigosen (1921), who made a study of the connective tissue cells of the chick embryo in film preparations, not only describes the accumulation of vacuoles in these cells but points out that certain granules described by other investigators (Albrecht, 1903; Renaut, 1907) are probably also abnormal, in view of the fact that these observers employed the film method. In cultures, however, vacuoles seldom appear before the 2nd or 3rd day. In certain media their appearance was delayed

indefinitely, while in others the vacuoles accumulated in the cells until the latter took on a frothy or moth-eaten appearance as described by Maximow (1916) and W. H. Lewis (1919). It is difficult to determine the nature of the vacuoles as they occur under so many conditions; *i.e.*, starvation, degeneration, phagocytosis, lack of oxygen, influence of bacteria, and lack of dextrose. They formed in cells in what would seem to be a most nutritive medium, such as a plasma clot, and were lacking in cells in a medium composed only of salts and dextrose. They certainly seem to indicate the action of some deleterious influence, since, in almost every instance, the accumulation of vacuoles was followed shortly by the death of the cell.

The granules were present much less frequently than were the vacuoles. They never formed in cells in film preparations, but were often observed in cells in plasma cultures and were especially abundant in those grown in white of egg, as was shown in a previous communication (1921). They seemed to be material stored up by the cell attributable to the protein nature of the environment since they were observed only in media containing this substance.

Vacuoles may occur in cells which contain granules and, as previously shown, are especially prone to do so in cells in egg albumin. The granules and vacuoles found in plasma cultures have occasionally been mistaken for different phases of the same body (Maximow, 1916; Shipley, 1919), but this is undoubtedly not always the case, for, as has been shown in regard to cells in egg albumin (1921), the vacuoles may form in regions removed from that occupied by the granules and also, as will appear from these investigations, the formation of vacuoles may be prevented by the addition of dextrose to the medium.

Effects of Lack of Dextrose upon the Cells of Tissue Cultures.

The effect of the lack of dextrose upon the cells of tissue cultures was definite and pronounced, and inevitably resulted in the production of vacuoles. 212 cultures were prepared in various media lacking dextrose, and in every instance vacuoles were formed, after which the cells rapidly degenerated. On the other hand, media such as white of egg, amniotic fluid, and allantoic fluid, otherwise unsatisfactory for cultures, became favorable for the growths when a small amount of dextrose was added.

The lack of dextrose was especially detrimental to cultures grown in Locke's solution or in Locke's solution to which 15 per cent of chicken bouillon had been added. In these solutions without dextrose the cells became full of vacuoles and died within a few days, while in the same solutions containing dextrose they survived many days, depending upon the amount of this substance in the medium. Surprising to say, cells in solutions rich in protein but lacking in dextrose died much sooner than those in a simple salt solution containing dextrose.

Effects of Dextrose upon the Cells of Tissue Cultures.

Only cultures in Locke's solution containing 15 per cent chicken bouillon were used in these experiments, partly because the cells remain more normal in this solution and partly because of the difficulty of attempting to add definite percentages of dextrose to some of the other media.

When only 0.25 per cent dextrose had been added to the medium the cells grew luxuriantly but the formation of vacuoles was delayed for only a few days. The cultures were quite variable. In some the vacuoles developed after 3 or 4 days, while others remained normal for 6 or 8 days. All of the cultures (over 100) in this solution, however, did eventually exhibit vacuoles. These cultures seldom lived more than 10 days. While, as stated above, no attempt was made to add dextrose to the plasma medium, nevertheless it seems apt to mention in this connection that the vacuoles that appear in plasma cultures probably are attributable to the small amount of dextrose (supposedly about 0.25 per cent) which the plasma contains. Cultures that are frequently retransplanted into fresh plasma have a much larger amount of dextrose available and therefore remain normal for longer periods of time.

Cultures in media to which 0.5, 0.75, and 1 per cent dextrose had been added differed little in appearance. The growth in all these media was extensive, full of cell division, and survived from 2 to 4 weeks without forming vacuoles. In some of these preparations, after 2 weeks of healthy growth, the cells one after another rounded up and sank to the bottom of the hanging drop until, within the course of 3 weeks or a month, all the cells had degenerated. These

cultures died without forming vacuoles, the last cell being as normal in structure as those of the initial growth. In other preparations, after an interval of healthy growth, all of the cells rapidly formed vacuoles and all degenerated within the following day or two.

When larger quantities (2 to 5 per cent) of dextrose were added to the solution, the growth sometimes was not extensive, and in most instances it did not survive so long as that in the normal solution or in one containing 1 per cent dextrose, but these cells never contained vacuoles. The results obtained when such large quantities of dextrose were added were influenced by the change in hydrogen ion concentration of these cultures.² Cultures in normal Locke-Lewis solution, which has an initial hydrogen ion concentration of 6.8 or 7, had usually, upon degeneration, become pH 7.2 or 7.4. Those in media without dextrose had about the same final hydrogen ion concentration. On the other hand, the cultures in media containing as much as 5 per cent dextrose were distinctly acid (pH 5.6 to 6) when tested at degeneration, regardless of the initial hydrogen ion concentration of the medium. The rapidity with which death of the cultures took place seemed to depend upon the change in hydrogen ion concentration. The appearance of these cells after death was usually quite different from that of cells in media containing less dextrose. They did not round up or sink to the bottom of the drop, but remained spread out on the cover-slip, keeping their outlines as though coagulated into skeleton forms.

While the condition which arises in tissue cultures when the amount of dextrose in the medium is varied is somewhat analogous to that in diabetes, it is by no means due to the same cause. The acid which is found in cultures is probably due to the breaking down of dextrose, while the acidosis exhibited by the diabetic is presumably due to the partial combustion of fats resulting in the formation of fatty acids.

² The details of these results are given in another publication with Felton, who investigated the hydrogen ion concentration of tissue cultures in this laboratory, and for this reason will not be repeated here (Lewis, M. R., and Felton, L. D., The hydrogen-ion concentration of cultures of connective tissue from chick embryos, *Science*, 1921, liv, 636).

SUMMARY.

It is not advisable to enter into a discussion of these findings at the present time, owing to the lack of experimental evidence as to the exact nature of the vacuoles. Regardless of the nature of these bodies or of what factors produce them, they are structures abnormal to the connective tissue cells. The lack of dextrose in the medium of tissue cultures leads to some condition distinctly detrimental to the cells, resulting in their vacuolation and death, even when the medium contains abundant protein material. The addition of small amounts (0.5 to 1 per cent) of dextrose to the medium delays the formation of vacuoles and prolongs the life of the culture. The addition of large amounts (2 to 5 per cent) prevents vacuolation of the cells, but so much dextrose usually leads to a change in the hydrogen ion concentration of the culture resulting in an acid condition which arises coincidentally with the degeneration of the cells.

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CONSTRUCTION OF AN OXYGEN CHAMBER FOR THE TREATMENT OF PNEUMONIA.

BY WILLIAM C. STADIE, M. D.

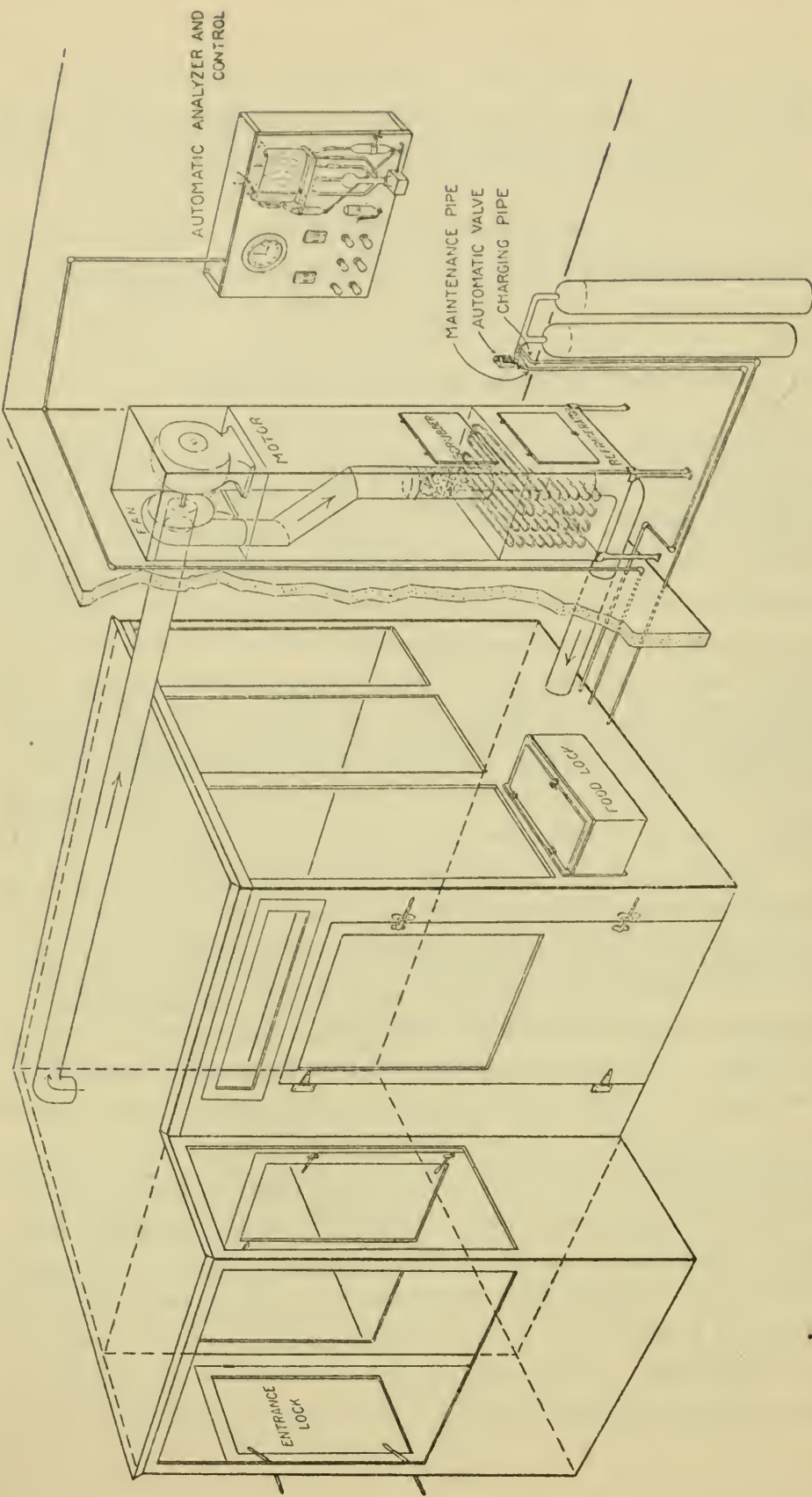
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PLATE 9.

(Received for publication, October 10, 1921.)

In the construction of a chamber or ward for the treatment of pneumonia patients, which is to be filled with an atmosphere with a higher oxygen content than ordinary air, there are several requirements which must be fulfilled. The chamber must be large enough to accommodate attendants as well as the patient, so that adequate nursing care may be given to acutely ill patients; it must be easily and quickly filled with oxygen to the desired concentration; this concentration must be maintained with facility and a minimum use of oxygen; the temperature of the room must be kept within comfortable limits, and the humidity, which may increase rapidly through the exhalation of moisture from the lungs of the patient and attendants must be kept low; the carbon dioxide excreted must be removed as formed. Moreover, the chamber must be easy of ingress and egress, especially so when acutely ill patients are treated, and above all it must be reasonably economical in the cost of operation. All these objects have been attained in the oxygen chamber described below. It is ready for the routine treatment of patients after a few minutes of preparation and may be run for days with a minimum of attention.

The oxygen chamber (Text-fig. 1 and Fig. 1) has been built in one of the wards of the hospital. It measures $10 \times 8 \times 8$ feet, length, width, and height respectively, and has a total capacity of 640 cubic feet, or 18 cubic meters. The filling, maintenance, cooling, and analyzing devices have all been placed in an adjoining small room for convenience and sightliness. The entire apparatus includes the following: the oxygen chamber proper, doors for entrance, entrance lock, food



TEXT-FIG. 1. Schematic drawing of oxygen chamber with ventilating system, carbon dioxide scrubber, cooling device, automatic oxygen analyzer, and filling and maintenance devices.

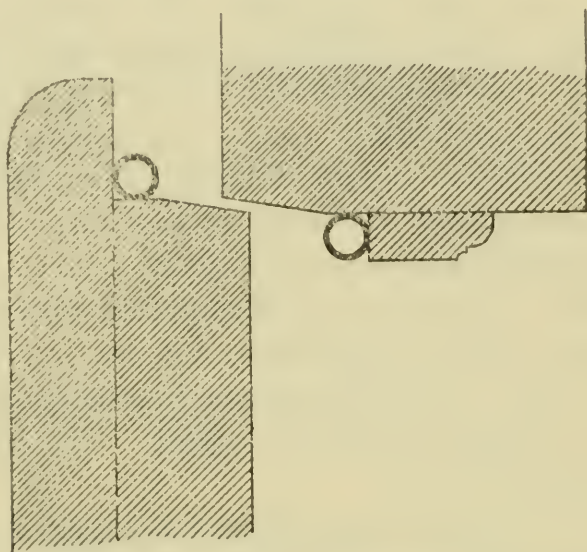
lock, ventilating system consisting of a fan, refrigerator, and carbon dioxide scrubber; the filling and maintenance apparatus consisting of oxygen tanks; a solenoid valve for the automatic regulation of the oxygen; an automatic oxygen analyzer and oxygen maintenance device; a carbon dioxide analyzer.

The chamber proper is built upon the tile floor of the main ward. Special precautions must be taken to insure gas tightness so as to minimize oxygen loss. The foundation consists of a heavy 4 inch base-board which is set upon a flat rubber gasket. Both rubber gasket and base-board are imbedded in a mixture of white lead and varnish, which makes a gas-tight joint with the floor. The lower part of the chamber consists of wooden panels, while the upper part consists of wooden sash work into which are set plate glass windows. The roof is likewise composed of panels. All seams have been covered over, both inside and outside of the chamber, by broad wooden strips set in a heavy layer of the white lead and varnish mixture. In addition, all corners have been filled in with quarter round molding similarly imbedded. The roof has been coated with a heavy pitch, and all grooves and crevices, both inside and out, have been carefully filled with white lead. Over all, four or five coats of paint with a finishing coat of enamel have been applied. The chamber proper is practically gas-tight.

The doors of the chamber have offered the greatest problem with respect to the gas tightness. Reference to Text-fig. 2 will show the type of construction used. A double offset on the door fits closely on similar offsets on the jamb. Two gaskets of soft rubber tubing are placed as shown. When the door is closed, two large refrigerator levers are forced down on the inclined plane of the hooks; a firm and even pressure is thus obtained, bringing both rubber gaskets in close contact with the door, making a practically gas-tight joint. The large door is used for the passage of the patient and the bed into the chamber. It is then closed for the duration of the treatment. Ingress and egress of attendants is made through a smaller door which is protected by an *entrance lock*. The entrance lock, which measures $3 \times 8 \times 5$ feet, with a total capacity of 120 cubic feet, also has a door of the same construction, and serves to minimize the escape of the rich oxygen mixture from the chamber proper into the ward when

the doors are opened. The lock, of course, has the same construction as the chamber.

The ventilating system is necessary if the temperature and humidity are to be kept at comfortable levels, and moreover, it gives a movement of the air in the chamber which makes the atmosphere more comfortable. By means of an 8 inch duct leading from the roof, the air in the chamber is drawn out by a *ventilating fan*. From the fan the air is conducted downward through a compartment or *scrubber* which was originally intended to be filled with material for the removal of carbon dioxide. The air then passes



TEXT-FIG. 2. Construction of door showing offsets and rubber gaskets.

through a large *refrigerator* which contains 50 feet of 1 inch piping in which there is an active circulation of cold brine. Here the air is cooled and the excess of moisture precipitated. The air then passes through a 6 inch duct back into the chamber where, to minimize drafts, it is deflected downward toward the floor.

Food Lock.—A small *food lock*, measuring $16 \times 24 \times 13$ inches, with a double door fitted with rubber gaskets attached to the side of the chamber is necessary for the passage in and out of the chamber of food and other small articles, thus obviating the necessity of frequent openings of the large entrance lock.

Communication into the chamber is obtained by telephone.

Filling the Chamber.—The oxygen is contained in large steel cylinders under 1,800 pounds pressure. Two tanks are coupled to a manifold or short piece of iron piping, and by means of a reducing valve the pressure is carried down to 0 to 100 pounds pressure. When the chamber is to be filled the oxygen is turned on as rapidly as possible through the reducing valve, and passes directly into the chamber by means of a *charging pipe* $\frac{1}{4}$ inch in diameter, the fan being run during the filling.¹

When the charging of the chamber is completed the maintenance of the desired percentage is accomplished automatically. Further admission of oxygen is through the *maintenance pipe* on which is a solenoid *valve* controlled automatically by the oxygen analyzer and automatic regulator.

Automatic Oxygen Analyzer.

This consists of two parts, the analyzer and the electrical controlling device. The operation of the analyzer can be best understood by reference to Text-fig. 3 and Fig. 2 and a description of one complete cycle of operation. The analyzer consists of a gas sampling absorbing

¹ It has been found that the filling of the chamber closely follows a curve derived as follows: A vessel whose volume is V , filled with air, has slowly added to it pure oxygen. Diffusion is assumed to occur instantaneously. Excess of the resulting mixture escapes at minute pin-hole leaks, each constituent in proportion to its percentage at the time. If x be the amount of gas (oxygen) added, and y the proportion of oxygen at any time, then if dx of oxygen be added, there escape ydx parts of oxygen and there remain $dx - ydx$.

Therefore, $\frac{dx - ydx}{V} = dy$, the increase in proportion of oxygen.

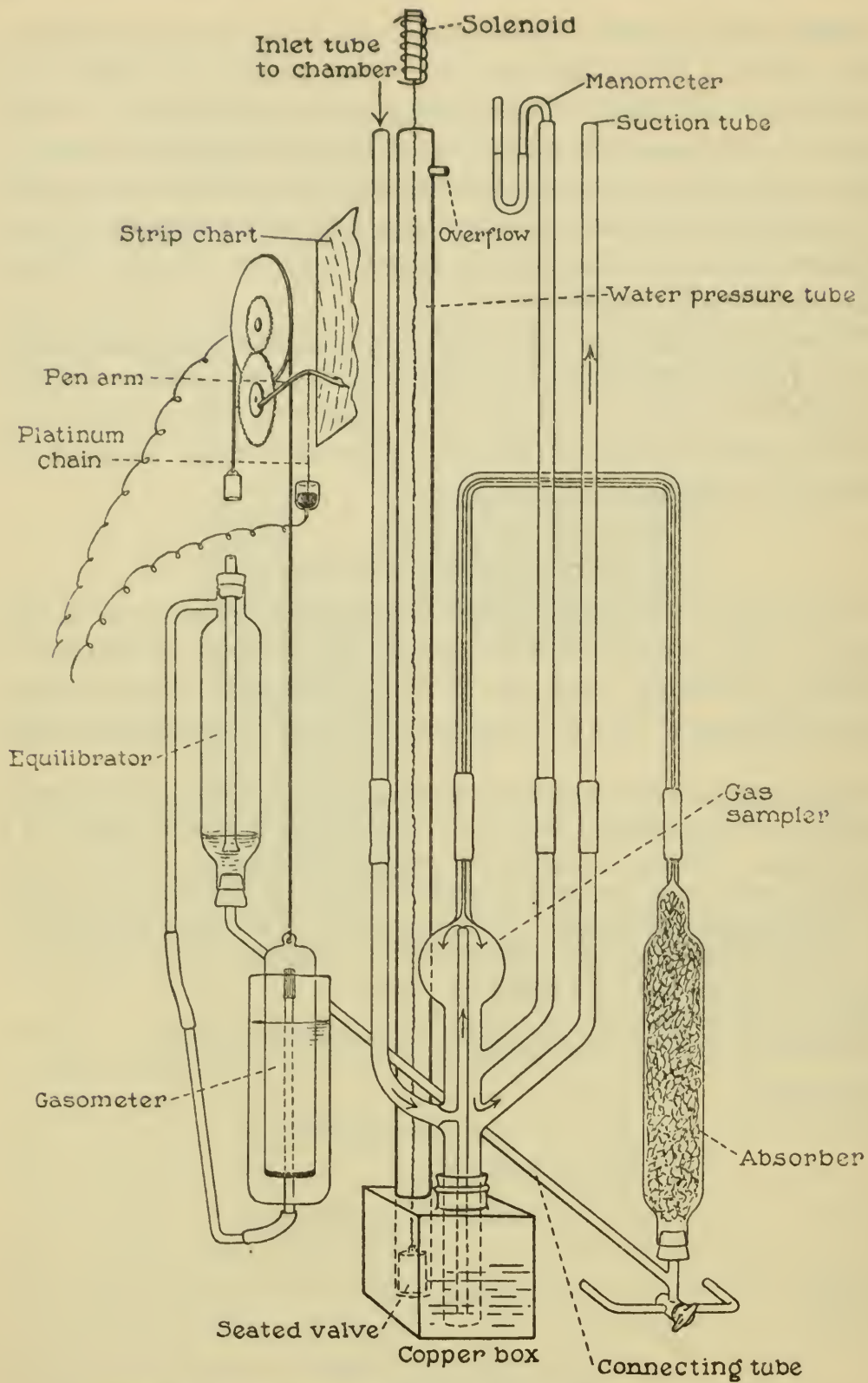
Integrating

$$\int dx = V \int_{y=0.2 \text{ (initial proportion)}}^{y=\text{proportion desired}} \frac{dy}{1-y}$$

we obtain

$$x = V \operatorname{Log}_e \frac{0.8}{1-y}$$

$$x = 2.303 V \operatorname{Log}_{10} \frac{0.8}{1-y}$$



TEXT-FIG. 3. Schematic drawing of automatic oxygen analyzer.

bulb, equilibrator, gasometer, meshed wheels, pen arm, and strip chart. The electrical controlling device comprises a clock fitted with contact points and wired to make two contacts every 20 minutes, relays, resistance lamps, and electrical connections to the house current and analyzer. A water vacuum pump is attached to the *suction tube*. By this means a sample of air from the chamber is drawn through the *inlet tube* and the *sampling bulb*. A constant stream of water runs into the *water pressure tube*. The water level slowly rises; when it reaches the height of the inlet and suction tubes, the suction is shut off by the water seal thus formed (the suction now operates through a by-pass, not shown, thus preventing the water and gas sample from being sucked out of the apparatus). A sample of gas is thus trapped in the sampling bulb. The manometer on the manometer tube instantly adjusts the pressure of the gas sample to atmospheric pressure as soon as the suction is shut off by the water seal formed. The water in the water pressure tube and apparatus continues to rise, and the sample of gas in the sampling bulb is pushed over into the *absorber*. The absorber is filled with fine copper wire gauze and a solution of ammonium acetate, ammonium hydroxide, and water.² The gas, as it passes into the absorber, displaces an equal amount of the solution which passes up through the connecting tube into the *equilibrator*. A corresponding volume of air in the equilibrator is forced into the *gasometer* which, being counter-balanced, rises and rotates the two meshed wheels, the second wheel carrying a stop-pin which, when the analysis is complete, limits the fall of the *pen arm*. The pen, fastened to a ratchet wheel, is independent of the other two wheels and is held up by a pawl acting on the fine milled ratchet. When the entire sample has been pushed out of the sampling bulb, the water does not rise any higher, but overflows into an *overflow pipe* on the water pressure tube. The gas in the absorbing bulb is allowed to remain in contact with the copper for 20 minutes, during which time all the oxygen is absorbed; and, as a result of this absorption, a certain amount of the solution siphons back into the absorber from the equilibrator. The gasometer sinks

² Ammonium acetate.....1,000 gm.
 Ammonium hydroxide (conc.)..... 600 cc.
 Water to2,000 cc.

correspondingly. When this absorption is complete, the clock makes the first contact and the small magnet below the gasometer pulls the string which lifts the pawl from the ratchet wheel. The pen then drops until it is stopped by the pin on the small wheel, and its position then corresponds to the amount of oxygen present in the sample. As it falls the pen writes a line on the moving strip chart, the lower point of which indicates upon the scale the percentage of oxygen present in the sample. The second contact is then made by the clock closing the circuit on the *solenoid valve* above the water pressure tube, and this solenoid magnet pulls up a *seated valve* in the copper box, allowing the water in the apparatus to run out. The fluid in the equilibrator then siphons back into the absorber and the gasometer sinks to its original level, rotating the two wheels and bringing the pen to its original position. The valve in the copper box then drops when the contact is broken by the clock, a new sample of gas being meanwhile taken in by the suction, the water again rises in the apparatus, and the operation is repeated.

In addition to analyzing and recording the oxygen percentage the apparatus also controls the solenoid valve on the maintenance pipe to the oxygen chamber above described. The pen carries a small fine *platinum chain* which, through the pen and its support, forms one leg of a circuit, the other leg being connected with the *mercury cup*. The mercury cup is set at any desired position corresponding to the proportion of oxygen desired. When an analysis is completed and the pen drops to record it, if it is less than the desired percentage the end of the platinum chain will dip into the mercury, thus closing the circuit on the solenoid maintenance valve (Text-fig. 1) which opens, allowing oxygen to pass into the chamber. The contacts are so arranged that this valve is kept open for about 2 minutes, during which time from 50 to 100 liters of oxygen may be admitted to the chamber. In this way 300 liters of oxygen may be admitted into the chamber every hour, which is greater than the usual loss. If, however, upon completion of an analysis the percentage indicated is greater than that for which the mercury cup is set, the platinum chain will not touch the mercury and the oxygen valve remains closed. The circuit in the clock and automatic oxygen control carries only currents of a few volts supplied by a battery of lamps

from the house current. These contacts control the relays which make and break the 110 volt circuit in the solenoid magnets which have lamp resistances in parallel. In this way heavy current is avoided on light parts and arcing of the contacts is prevented.

With this arrangement it has been found in practice that after the chamber has been charged and the automatic analyzer and regulator started, the oxygen chamber will maintain itself at any desired oxygen percentage from 40 to 60 per cent, with practically no attention except to renew the copper and solution in the analyzer twice a day.

The Removal of Carbon Dioxide.

This offers a serious problem from the point of view of efficiency and economy. It was originally planned to place in the ventilating system, as shown, a container with soda-lime to remove the carbon dioxide as it was formed. It was found, however, difficult to get soda-lime of sufficiently large mesh to offer little resistance to the passage of the air through the system. All of the samples tried, even soda-lime screening from 4 to 6, was found to offer so much resistance as almost completely to shut off ventilation. In practice this resulted in a rapid elevation of the temperature and humidity of the chamber to an uncomfortable degree. Moreover, the best grades of soda-lime are quite expensive. With these two difficulties in mind, it was important to find a substitute. A scrubber was designed for the use of the sodium hydroxide in solution, but this proved very inefficient. The product called natron, which is sodium hydroxide in thin shells, was found to be efficient, easy to handle, and economical. Natron is put out in 3×20 inch cylindrical cartons of paraffin cardboard, which have been designed for use in respiratory apparatus and carbon dioxide analyzers of flue gas. One of these cartons is placed on the outlet of a small blower fan which is placed in one corner of the chamber and the air of the chamber is blown through the column of natron. With two people in the chamber one carton will keep the carbon dioxide at practically zero for 2 to 3 hours. The cartons are changed very easily, it being simply necessary to cut out both ends of the carton with a sharp knife and place the fresh one in the blower. In this way the cost of carbon dioxide removal is

considerably reduced. Table I illustrates how rapidly and efficiently one natron carton placed on a small Buffalo Forge fan will remove the carbon dioxide.

TABLE I.
Removal of Carbon Dioxide by Carton of Natron.

Time elapsed.	Carbon dioxide.
<i>hrs.</i>	<i>per cent</i>
0	1.34
$\frac{3}{4}$	0.05

Water Removal.

In practice the refrigerator has been found quite efficient in keeping the temperature of the chamber down to about 65°F. and also in keeping the humidity between 40 and 50 per cent. Table II shows that even when water vapor is vaporized into the chamber at a rate 20 times the maximum for one person the humidity and temperature are kept within reasonable limits.

TABLE II.
Test of Refrigerator for Removing Water Vapor.

900 gm. of water vaporized into chamber in 1 hour.

Hr.	Time elapsed.	Temperature.	Humidity.
<i>p.m.</i>	<i>hrs.</i>	<i>°F.</i>	<i>per cent</i>
12.30	0	70	64
1.00	$\frac{1}{2}$	70	66
1.30	1	71	68
Control; ventilating system not running; office fan in chamber to mix air.			
4.00	0	72	45
4.15	$\frac{1}{4}$	76	53
4.25	$\frac{1}{2}$	75	87
5.00	1	76	82

Effect of Opening Doors.

Entrance into the chamber through the lock means, of course, a loss of oxygen. When the inner door is opened into the lock, part

of the oxygen from the chamber must pass into the lock. If equilibrium is attained, that is, if there is a rapid diffusion from the chamber into the lock so that the percentage in both is the same, then the decrease of percentage in the main chamber may be calculated from the formula

$$\text{Percentage loss} = \frac{V^1 P - 0.2 V^1}{V + V^1}$$

in which V^1 is the volume of the lock, V is the volume of the chamber, and P is the percentage of oxygen in the chamber. In actual practice the loss is less than this, due no doubt to the fact that when the doors are quickly opened and closed, equilibrium in the lock and chamber with respect to the oxygen percentage is not attained. This is shown in Table III.

TABLE III.

Loss of Oxygen from Oxygen Chamber on Opening Air Lock.

Oxygen in chamber.	Loss of oxygen.			
	Calculated.	Found.	Calculated.	Found.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>cubic meters</i>	<i>cubic meters</i>
40	3.6	1.1	0.65	0.20

Charging and Maintenance of Oxygen Chamber.

The amount of oxygen required to attain a given percentage of oxygen in the chamber varies, of course, with the percentage desired, and is given by the equation derived on page 327, from which the values in Table IV are calculated. The chamber was maintained usually at 50 per cent of oxygen and 8.6 cubic meters of oxygen are necessary to give this. The amount of oxygen necessary to maintain the oxygen percentage at this figure is small; Table V gives the rate of loss of oxygen in the chamber, ducts, and refrigerator with the ventilating fan running.

In actual practice, however, the loss is greater than this, due to the consumption of oxygen by the patient and nurse, which is about 0.025 cubic meters per hour, and the far greater loss due to the frequent opening of the air lock.

Table VI shows the amount of oxygen required during an actual treatment of a pneumonia patient.

TABLE IV.

$$\text{Calculated Amount of Oxygen} \left(x = 41.5 \log_{10} \frac{0.8}{1-y} \right)$$

Required to Charge Oxygen Chamber to a Given Percentage.

Oxygen	Volume of oxygen required
<i>per cent</i>	<i>cubic meters</i>
30	2.4
40	5.2
50	8.6
60	12.6
70	17.8

TABLE V.

Maintenance Test.

Oxygen chamber charged with 4.9 cubic meters of oxygen. Calculated oxygen = 40.0 per cent. Ventilating fan running. No patient in chamber. Doors not opened.

Time elapsed.	Oxygen.	Loss per hr.	Loss per hr.
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>cubic meters</i>
0	39.8		
$\frac{1}{4}$	39.7		
$\frac{1}{2}$	39.6		
1	39.2		
$3\frac{1}{4}$	38.7		
13	35.5	0.33	0.060

TABLE VI.

Amount of Oxygen Required for Charging and Maintaining of Oxygen Chamber during Treatment of Pneumonia Patient.

Patient.	Duration of treatment.	Oxygen maintained.	Amount of oxygen required to charge chamber.	Amount of oxygen required for maintenance.	Loss per hr.
	<i>hrs.</i>	<i>per cent</i>	<i>cubic meters</i>	<i>cubic meters</i>	<i>cubic meters</i>
U.	24	40-50	9.3	4.0	0.17
U.	17	50-60	9.4	2.0	0.14
Total.....	41		18.7	6.0	0.31
			Grand total..... 24.7		

SUMMARY.

1. The construction of an oxygen chamber is given. This chamber can be quickly filled with oxygen to any concentration up to 65 per cent and maintained at the desired concentration for an indefinite time.

2. The construction of ventilating system, cooling device, carbon dioxide remover, automatic oxygen analyzer, and filling and maintenance devices is given.

3. The chamber is designed so that pneumonia patients with anoxemia may be placed in it and breathe an atmosphere containing 40 to 60 per cent of oxygen.

4. The chamber is easy of ingress and egress, is economical in cost of operation, and comfortably accomodates patient and attendants so that adequate nursing and medical attention can be given at all times.

EXPLANATION OF PLATE 9.

FIG. 1. Photograph of oxygen chamber in use to show size relative to patient and attendants.

FIG. 2. Photograph of automatic oxygen analyzer with electrical controlling device.



FIG. 1.

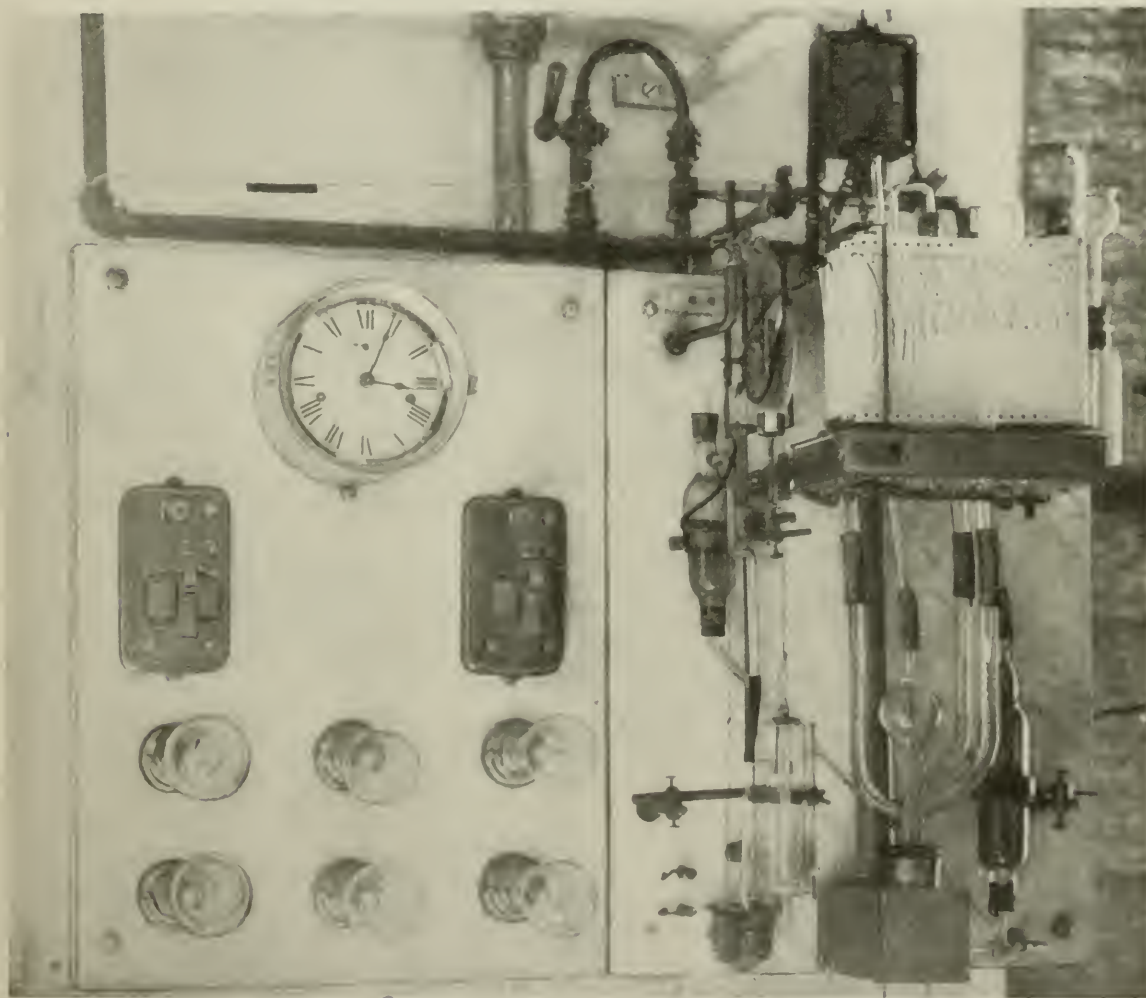


FIG. 2.

(Stadie: Oxygen chamber for treatment of pneumonia.)

THE TREATMENT OF ANOXEMIA IN PNEUMONIA IN AN OXYGEN CHAMBER.

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Shortly after the discovery of oxygen, and particularly after the demonstration by Lavoisier of oxidation as a life process, oxygen came into use as a therapeutic agent. It has been given by inhalation, by subcutaneous and intravenous injection (Stuertz, 1903; Tunncliffe and Stebbing, 1916), and by rectal insufflation. In addition, ozone also has been extensively used by inhalation, and hydrogen peroxide given intravenously in cases of influenzal pneumonia (Oliver and Murphy, 1920). Oxygen itself has been used in the treatment of a great variety of diseases; the literature which has grown up about its use is extensive; the opinions regarding its efficacy and the conditions under which it should be used are varied. Little would be gained by a review of this literature here; nevertheless, it is important to state the reasons for its use in disease, giving particular emphasis to the therapeutic use of oxygen in the treatment of pneumonia patients.

The use of oxygen as a therapeutic agent is rational only when, by reason of a disturbed metabolism or an insufficient oxygen supply, either local or general, there exists a condition of suboxidation. This may be either acute or chronic, and presumably results in harm by virtue of the fact that intracellular processes are disturbed by diminished oxidation or accumulations of unoxidized products of cell activity. There are many causes of suboxidation, but the one which interests us chiefly here is commonly called anoxemia. The term anoxemia as ordinarily used means "hypo-oxemia," or a condition in which there is a diminished amount of oxygen in the circulating blood; hence anoxemia is more correctly defined as that condition in which the hemoglobin of the blood is less saturated with oxygen than normally. It must not be assumed that anoxemia necessarily pre-

supposes a condition of suboxidation, for the relations between normal function and function under varying degrees of anoxemia are not definitely established. It will suffice to point out here why it is usually assumed that anoxemia results in a condition of suboxidation and is therefore deleterious to the organism.

Oxygen Tension of the Blood and Tissue Tension.

In considering the passage of oxygen from the arterial blood to the tissues two factors must be recognized: normal blood has available for tissue respiration about 20 volumes per cent of oxygen (capacity factor) at a tension (intensity factor) ranging from 100 to 0 mm. Hg. The average normal amount of oxygen taken by the tissues from the arterial blood is 6 volumes per cent, or 30 per cent of the total capacity. The dissociation curve of average human blood (Barcroft, 1914) shows that when the arterial blood is completely saturated, the 6 volumes per cent will be delivered to the tissues at a tension greater than 35 mm. Hg. In other words, the amount of oxygen ordinarily used is available at this relatively high tension. The remaining 16 volumes must be given to the tissues at relatively low tension (less than 35 mm. Hg.).

If, however, the blood in the arteries is only partially, *e.g.* 70 per cent, saturated, there are still available for the tissues 14 volumes per cent of oxygen. This is more than enough for ordinary purposes. But this oxygen is at a tension less than 35 mm. Hg. A person with this degree of anoxemia is in extreme distress. Although the oxygen of his blood is abundant in amount, it is available at low pressures only; so that unless it is assumed that the tension at which the oxygen is available for tissue metabolism is of as much importance as the total amount, it is difficult to understand how such a condition of anoxemia can be harmful.

There is but little and inconclusive evidence, however, to show that the tension of oxygen in the blood in contradistinction to the total amount available is important. Verzár (1912) and later Krogh (1919) have demonstrated that, contrary to previous conceptions, the oxygen tension of the tissues is not zero, but between 30 and 40 mm. Hg.; this would indicate that the tissues ordinarily *do* not,

although it does not show that they *may* not, function at low oxygen tensions. Vernon (1906) has studied metabolism of tissues under varying oxygen tensions, and his results, while far from conclusive, seem to indicate that tissue metabolism is not normal when the oxygen tension falls below a certain minimum. Verzár (1912), on the other hand, obtained contradictory results on different tissues, although his results on muscle, the functionally simplest tissue he used, showed that there is a striking decrease in oxygen consumption at low blood oxygen tensions. Burrows (1917) likewise, in studying the growth of tissue cells, concluded that whereas high oxygen tension is without influence, growth does not occur when the oxygen tension falls below that corresponding to the average oxygen tension of the venous blood of mammals.

The condition of anoxemia, resulting from carbon monoxide poisoning, is explainable also as due to a diminished tension of the blood oxygen. Presumably carbon monoxide itself has no poisonous action, and its harmful effects are due entirely to the temporary destruction of the oxygen-carrying power of the blood. This by itself would be difficult to understand, for saturation of as little as 50 per cent of the blood with carbon monoxide is serious, and saturation of 80 per cent of the hemoglobin is always fatal. Yet, in the first case there would still be in the average individual about 10 volumes per cent of oxygen available, and in the second case there would still be 4 volumes of oxygen available, which, as Lundsgaard (1919) has shown in cases of pernicious anemia, is about the lowest possible value compatible with life. Haldane (1912), however, has shown that in blood but partially saturated with carbon monoxide the oxygen dissociation curve of the available hemoglobin is markedly modified. The initial half of the curve is much steeper and the second half much flatter than normally, so that at low tension of oxygen the blood is more saturated with oxygen than normal blood, but, conversely, holds on to its oxygen more tenaciously in the capillaries. Reference to the curve will show that in a blood half saturated with CO only 2 volumes per cent of the available 10 volumes of oxygen will be at a tension above 50 mm.; the remaining oxygen will be at a lower partial pressure. From this Haldane concludes that anoxemia of carbon monoxide poisoning is due to a marked diminution of the tension of the oxygen of the blood.

Although this experimental evidence is far from conclusive, clinical experience gives suggestive support to it. Congenital cardiac disease is associated with chronic anoxemia, and it is possible that the mental and physical deterioration seen in these cases is due in part at least to anoxemia. One case under our observation had an oxygen capacity of 25 volumes per cent, the arterial blood being 80 per cent saturated. Despite the 20 volumes per cent of available oxygen in the arterial blood, typical signs of physical and mental deterioration were present. It would be difficult to understand how anoxemia could play any rôle in this condition unless the tension of oxygen in the blood is important.

In this connection, however, we must consider the fact that under certain conditions the body may use all the oxygen in the blood. In other words, the oxygen, even at the lowest tension, is utilizable. When the arm is exposed to cold the venous blood may contain little or no oxygen (Meakins, 1921). Again, Lundsgaard (1919) showed that in cases of pernicious anemia in which the oxygen capacity was as low as 5 to 6 volumes per cent the venous blood from the arm was almost completely deprived of oxygen, and he concluded that: "The last residuum of the oxygen in the blood . . . is taken away by the body cells in resting individuals just as easily as the first part." In the postmortem heart's blood of pneumonia cases Stadie (1919) found the blood as little as 5 per cent saturated.

On the other hand, while certain tissues may use oxygen at low tensions it does not follow that their function is normal, and it may well be that other tissues, particularly the kidney and the central nervous system, show disturbed function with deficient oxygen tension. Lutz and Schneider (1919) and Ellis (1919) showed that the respiratory center is quite sensitive to comparatively slight changes in oxygen tension, and recently Doi (1921) has confirmed this in cats. Haldane (1919) in experiments upon himself showed marked mental disturbances under an oxygen tension of 40 mm. His arterial blood would be then about 75 per cent saturated and still had 13.8 volumes per cent of blood oxygen available. This amount, while more than normally necessary, was at a low tension, and hence presumably not available for the central nervous system. Meakins and Davies (1920) report an experiment in which the subject by breathing low oxygen

mixtures reduced the arterial saturation to 81.4 per cent. Headache and general malaise resulted.

In striking contrast to the distress which follows anoxemia when the oxygen in the blood is abundant but at low partial pressures are cases of anemia. With as little as 6 to 8 volumes of oxygen per 100 cc. of blood these patients are quite comfortable at rest (Lundsgaard, 1919). The blood here is completely saturated, and hence the oxygen, while small in amount, is at a high pressure.

A well known response to anoxemia is an increase in the oxygen capacity of the blood. This is observed in pneumonia with cyanosis, congenital and acquired cardiac disease, as well as after exposure to high altitudes. In this way the amount of oxygen supplied at all tensions is increased. At any given percentage saturation the blood will give up more oxygen than normal blood so that this mechanism compensates in part for the partial saturation of the blood in the arteries.

Use of Oxygen in Pneumonia.

In pneumonia there frequently occurs a condition of anoxemia. While there is no direct evidence to show that this acute anoxemia, often of profound degree, is harmful, nevertheless it is usually assumed that the presence of anoxemia is dangerous. In a series of thirty-three pneumonia cases (Stadie, 1919) there was only one case which recovered in which the arterial unsaturation of the blood was greater than 20 per cent. A high degree of anoxemia in pneumonia, then, is accompanied by a high mortality, and yet it must be distinctly remembered that they are not necessarily cause and effect, since the degree of anoxemia varies directly with the severity of the infection and the extent of the consolidation. It is possible then that the anoxemia is simply a concomitant feature of intense and extensive infections and plays no rôle in the ultimate fatality. Not until the relation of function to oxygen tension is further elaborated can it be definitely said that an anoxemia *per se* is a factor in the fatal outcome.

Since anoxemia is a frequent and often a pronounced symptom of pneumonia, a study of the effects of oxygen upon this type of anoxemia and upon the course of the pneumonia was begun and is here reported. The anoxemia is due to an insufficient aeration of the

blood in its passage through the lungs. As to the mechanism of this deficient aeration, it is usually assumed that the consolidation of part of the lung, the presence of many small patches of infiltration extending from the main or initial focus, the plugging of many small bronchi, and the coating of the alveoli with exudate and moisture diminish the respiratory surface or hinder the diffusion inward of oxygen. This explanation does not stand alone, and recently Meakins (1920, *a*) states that: "The anoxemia occurring in acute lobar pneumonia is the result of the rapid and shallow breathing typical of this condition" which purely mechanically lessens ventilation of the alveolar spaces. In both cases the administration of oxygen would tend to relieve the anoxemia by greatly increasing the percentage of oxygen in the alveolar air and hence its diffusion pressure.

In critically ill cases of pneumonia, then, it is conceivable that anoxemia might make serious inroads upon the resistance of the patient and hasten the end. Certainly experience has shown that cases with an arterial unsaturation greater than 20 per cent usually proceed to a rapid and fatal termination. In these cases the relief of anoxemia might prolong life until the forces of immunity could assert themselves.

The cases here reported were treated with oxygen in an oxygen chamber described in the accompanying paper (Stadie, 1922). By this means the treatment could be continued over a prolonged period of time, the percentage of oxygen administered fixed at any desired level, discomfort and inconvenience of masks, funnels, and tubes eliminated, adequate medical and nursing attention rendered at all times, and, moreover, accurate observations of the blood and respiration made under exactly known conditions. Eight cases are here reported. All had a high degree of anoxemia and presented grave prognoses.

EXPERIMENTAL.

Eight cases from the pneumonia service of the Hospital of The Rockefeller Institute were treated from 1920-21. The cases treated were selected from all the pneumonia cases during this time. The mode of selection was as follows: All cases were carefully followed as to the degree of anoxemia present from time to time. The presence

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and extent of the anoxemia were estimated by determination of the arterial unsaturation. The extent of cyanosis, particularly in the finger-tips, also furnishes a valuable guide. When a case developed anoxemia of sufficient degree, usually 20 per cent or more of arterial unsaturation, oxygen treatment was commenced. The cases were followed by repeated determinations of the arterial unsaturation. When it was not feasible to do arterial punctures, estimates of the degree of anoxemia were made from the extent of the cyanosis. In order to compare cyanosis from time to time the following scale was used: \pm indicates very slight, + slight, ++ moderate, +++ marked, and ++++ intense cyanosis. Experience enables an observer to estimate closely, in this way, the degree of arterial unsaturation, as controlled by actual blood analysis. The case reports follow.

Case 1.—D. G., male, age 25 years. Admitted Mar. 25, 1921.

Present Illness.—Onset Mar. 21, with severe abdominal pain, nausea and vomiting, fever, headache, prostration, and chills.

Physical Examination.—Temperature 104.5°F.; pulse 160; respirations 36. No cyanosis. The condition of the lungs is shown in Text-fig. 1. Rest of physical examination negative.

Mar. 28. Patient is quite toxic. Has developed hiccough. Lung signs are as diagramed in Text-fig. 1. In addition to the area of consolidation on the right side there are many fine râles on the left side. Pulse rather poor in quality. Cyanosis +. 2 p.m. Patient is much worse than this morning. Cyanosis +++. Arterial unsaturation 37 per cent. There are many moist râles involving both sides. Oxygen treatment started.

Mar. 29. Patient's condition much improved. He is still delirious. Has continual hiccough. Cyanosis absent. Arterial unsaturation 3 per cent. Lung signs about the same as yesterday. 80 cc. of sterile fluid withdrawn from right chest.

Mar. 30. During his stay in the oxygen chamber the patient appeared much improved. There were extra systoles. Removed from the chamber last night, since which time he has been becoming worse. Respirations more rapid and irregular; hiccough at every respiration. Cyanosis has increased markedly. Arterial unsaturation 22 per cent. Evidence of consolidation of entire right side. There are coarse moist râles throughout both lungs. 4.30 p.m. Patient is distinctly worse and the outlook is very doubtful. Cyanosis is extreme. Arterial unsaturation about 35 to 40 per cent, estimated from degree of cyanosis. Oxygen treatment commenced. 10 p.m. Cyanosis has entirely cleared up. Hiccoughs continue and are extremely prostrating.

Mar. 31. No cyanosis. Arterial unsaturation 1.5 per cent. All efforts to relieve hiccough without avail.

Apr. 1. Lung signs as shown in Text-fig. 1. Hiccough still continues. No cyanosis.

Apr. 3. Temperature is almost normal. Cyanosis +. Hiccoughs still persist.

Apr. 5. Patient stopped hiccoughing quite suddenly. He had hiccoughed without interruption for 9 days. For this reason he was not taken out of the oxygen chamber sooner. Convalescence uninterrupted.

Bacteriological Examination.—Type II pneumococcus recovered from the sputum. Blood cultures negative.

Discussion.—When the impairment of the respiratory surface had extended to the opposite side of the initial lesion, as shown by the presence of râles, cyanosis became much more marked. The anoxemia disappeared under the effect of oxygen. A short period without oxygen during the course of the illness resulted in a return of the marked anoxemia, so that the patient's condition looked quite desperate. This anoxemia was again relieved by oxygen. Recovery followed.

Case 2.—E. K., male, age 25 years. Admitted Feb. 3, 1920.

Present Illness.—Acute onset 1 week ago with malaise, headache, body pains and cough, and blood-tinged sputum.

Physical Examination.—Negative except for lungs. Slight dullness at left lower back. No râles. No cyanosis.

Feb. 4. Lung signs as diagramed in Text-fig. 2. No cyanosis.

Feb. 6. Definite signs of consolidation of entire right lower lobe. Many coarse musical râles on the right side. Patient has a definite cyanosis (+).

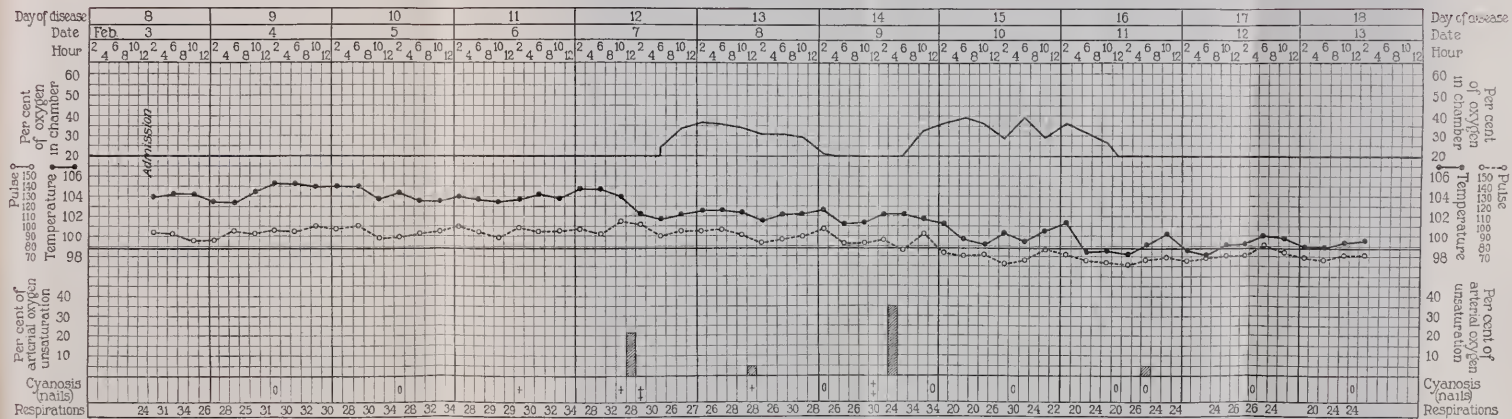
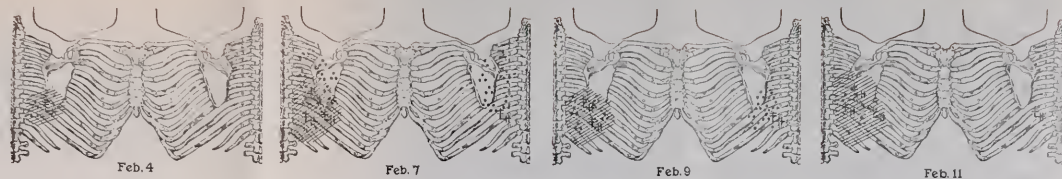
Feb. 7. The patient is definitely worse this morning. Cyanosis is more marked. Patient delirious. Lung signs are as diagramed in Text-fig. 2. Arterial unsaturation 22 per cent. Patient is extremely ill and prognosis is grave. Oxygen treatment started.

Feb. 8. Patient has been in the oxygen chamber since yesterday at 4.30 p.m. Cyanosis is distinctly less. Arterial unsaturation has decreased to 5 per cent. Lung signs about the same as yesterday.

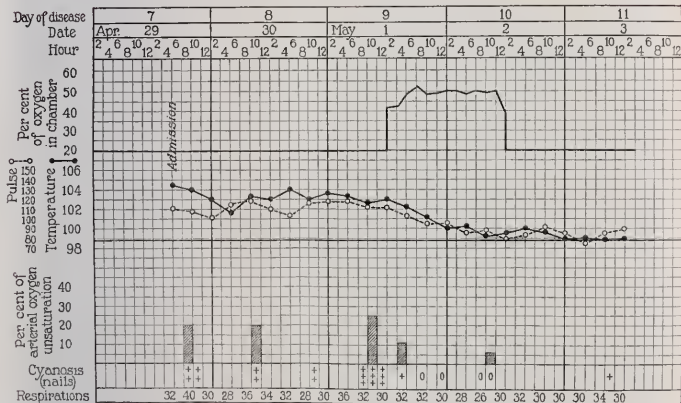
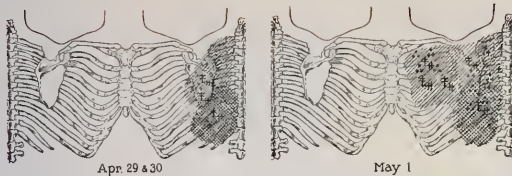
Feb. 9. Condition much better than on Feb. 7. Cyanosis has disappeared. Mentally he is more alert. Signs are as indicated in Text-fig. 2. Patient was removed from the oxygen chamber at 3 a.m. but soon relapsed into a critical condition. Cyanosis gradually returned until at 2 p.m. there was marked (+++) cyanosis and arterial unsaturation had increased to 35 per cent. The patient looks desperately ill. Oxygen treatment recommenced.

Feb. 10. Patient had marked jaundice. Lung signs much the same as yesterday. Cyanosis has entirely disappeared.

Feb. 11. Patient's condition much improved. The lung signs have cleared up in that extensive râles on the right and left sides have disappeared, there being signs of consolidation of the right lower lobe only. Oxygen treatment discontinued. No cyanosis. Arterial unsaturation 5 per cent. Convalescence uneventful.



TEXT-FIG. 2. Case 2.



TEXT-FIG. 3. Case 3.

Bacteriological Examination.—Type IV pneumococcus recovered from the sputum. Blood cultures negative.

Discussion.—At first the patient had slight signs which greatly increased until there was consolidation of the entire right lower lobe. Patient became more and more toxic. Cyanosis developed as lung signs increased, and with the appearance of extensive moist râles throughout both lungs, anoxemia became marked. Anoxemia was completely relieved by the administration of 40 per cent of oxygen. When the oxygen was withdrawn while the patient still showed evidence of extensive impairment of the lungs, his condition became much worse and the anoxemia advanced rapidly to 36 per cent. Readministration of oxygen relieved the anoxemia completely. Recovery followed.

Case 3.—H. B., male, age 41 years. Admitted Apr. 29, 1920. Chief complaint pain in chest and cough; duration 6 days.

Present Illness.—Onset Apr. 23, with sharp pain in the chest and chill, and cough with blood-tinged sputum. Patient delirious for the past 24 hours.

Physical Examination.—Patient quite sick. Marked cyanosis. Arterial unsaturation 20 per cent. Lung signs as diagramed in Text-fig. 3. Extensive consolidation of left side.

Apr. 30. Signs practically the same as yesterday. Cyanosis and arterial unsaturation the same.

May 1. Patient is definitely worse this morning. Spread of lung signs as shown in Text-fig. 3. Cyanosis (+++) more marked. Arterial unsaturation has increased to 25 per cent. Oxygen treatment started at 12 noon. 4 p.m. Cyanosis now only +; arterial unsaturation 12 per cent. 12 midnight. Patient is now pink, in striking contrast with the deep purplish cyanosis of this morning.

May 2. No cyanosis. Arterial unsaturation 6 per cent. Patient looks and feels much better. Temperature decreased to normal. Oxygen treatment discontinued. Convalescence uneventful.

Bacteriological Examination.—Type II pneumococcus from the sputum. Blood cultures negative.

Discussion.—Patient had extensive infection, with anoxemia lasting for a long period of time. Signs involving the entire left side with moist râles. Cyanosis became much more marked (+++). Treatment with oxygen caused rapid disappearance of cyanosis and anoxemia. Condition rapidly became normal.

Case 4.—J. V. S., male, colored, age 20 years. Admitted Mar. 4, 1921. Chief complaint cough and pain in the side.

Present Illness.—The day before admission the patient had a severe chill, headache, and developed a cough and bloody sputum. There was severe pain in the right side.

Physical Examination.—Negative except for lungs. Left lung normal. Right lung: Dullness from the fourth rib to the base; bronchial breathing with fine moist râles.

White blood corpuscles 20,600; polymorphonuclears 92 per cent.

Mar. 7. The patient ran an even course up to today. About + cyanosis and 15 per cent arterial unsaturation (Text-fig. 4). Today he is much sicker. Cyanosis has increased (+++). Arterial unsaturation has advanced to 35 per cent. Lungs show an extensive consolidation which involves the entire right lower lobe.

Mar. 8. Patient extremely toxic. Respiration rapid and labored. There is ++ cyanosis and 20 per cent arterial unsaturation. Patient is distinctly worse than yesterday, respirations being as high as 50 to 60. Oxygen treatment started at 12 noon. By evening the cyanosis had disappeared and arterial unsaturation had dropped to 5 per cent.

Mar. 9. Patient is somewhat better than yesterday. There are signs indicating extension of the process to the left lower base. No cyanosis. Arterial unsaturation 7 per cent. 8 p.m. Patient's temperature has receded, and although he still appears quite ill oxygen treatment is discontinued. The patient ran a prolonged course with evidence of consolidation of both lower lobes. Although the toxemia disappeared in great measure, the fever continued and the temperature did not reach normal until the 25th day. Convalescence then was uninterrupted.

Bacteriological Examination.—Pneumococcus Type II from the sputum. Blood culture negative.

Discussion.—The patient had a pronounced degree of anoxemia for a considerable period of time, at one time as high as 35 per cent of arterial unsaturation. Simultaneously with the spread of the pneumonia to the opposite side the patient became sicker. Under the influence of oxygen the anoxemia cleared up and concomitantly there was a fall in temperature and diminution in the severity of the toxic symptoms.

Case 5.—G. F., female, colored, age 17 years. Admitted Feb. 15, 1921. Chief complaint headache and pain in left side.

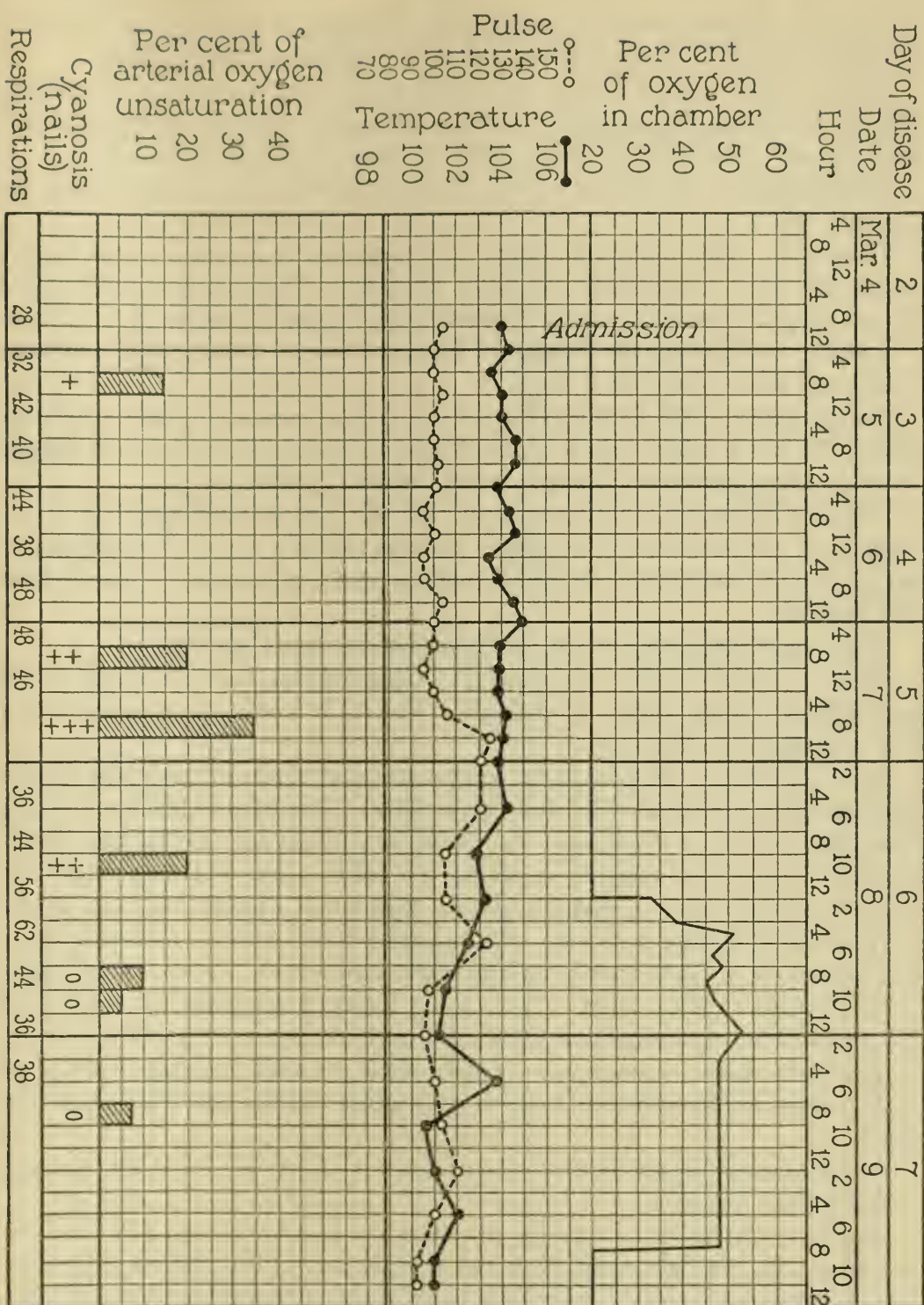
Present Illness.—Onset Nov. 9, 1920, with severe headache and cough, and pains in the left chest followed by nausea and vomiting. Scanty sputum.

Physical Examination.—Temperature 104.5°F.; pulse 140; respirations 50. Lungs: Dullness on the left side from the fourth rib to the base. Over this area there is bronchial breathing. Scattered râles over both lungs.

Feb. 17. Patient has been quite delirious during the night. Definite consolidation of the left lower lobe. Scattered râles throughout both lungs. Cyanosis ++ with 22 per cent arterial unsaturation (Text-fig. 5).

Feb. 18. The patient is still quite sick. Cyanosis (+++) has increased and arterial unsaturation is about 25 to 30 per cent estimated. 4 p.m. Oxygen treatment started.

Feb. 19. Oxygen treatment continued. Cyanosis has disappeared. Lungs: Complete consolidation over the entire left side with scattered moist râles both front and back. Scattered fine râles over right side. No cyanosis during the day.



TEXT-FIG. 4. Case 4.

Feb. 20. Patient is still extremely toxic. Lung signs as yesterday with scattered fine and medium sized moist râles over both sides. No cyanosis.

Feb. 21. The patient had a very good night and temperature is receding. Oxygen treatment discontinued yesterday afternoon. Patient had profuse perspiration and temperature fell by crisis. Convalescence uninterrupted.

Bacteriological Examination.—Type II pneumococcus was recovered from the patient's blood.

Discussion.—This was an intensely toxic patient with a process involving the entire left side. Considerable degree of anoxemia. The anoxemia was relieved by the oxygen which had no other apparent effect upon the course of the disease.

Case 6.—H. S., male, age 59 years. Admitted Mar. 1, 1921. Chief complaint cough and fever; duration 2 days.

Present Illness.—Up to time of onset the patient was feeling well. Feb. 26, 1921. He had a sudden attack of severe pain in the left side and then developed a severe cough productive of large amounts of thick, yellow sputum.

Physical Examination.—Temperature 103° F.; pulse 100; respirations 28. Lungs: Right side clear. Left lung: Dullness over the upper lobe; exaggerated breath sounds anteriorly. Scattered medium moist râles over left upper lobe. No cyanosis.

White blood corpuscles 24,000; polymorphonuclears 92 per cent.

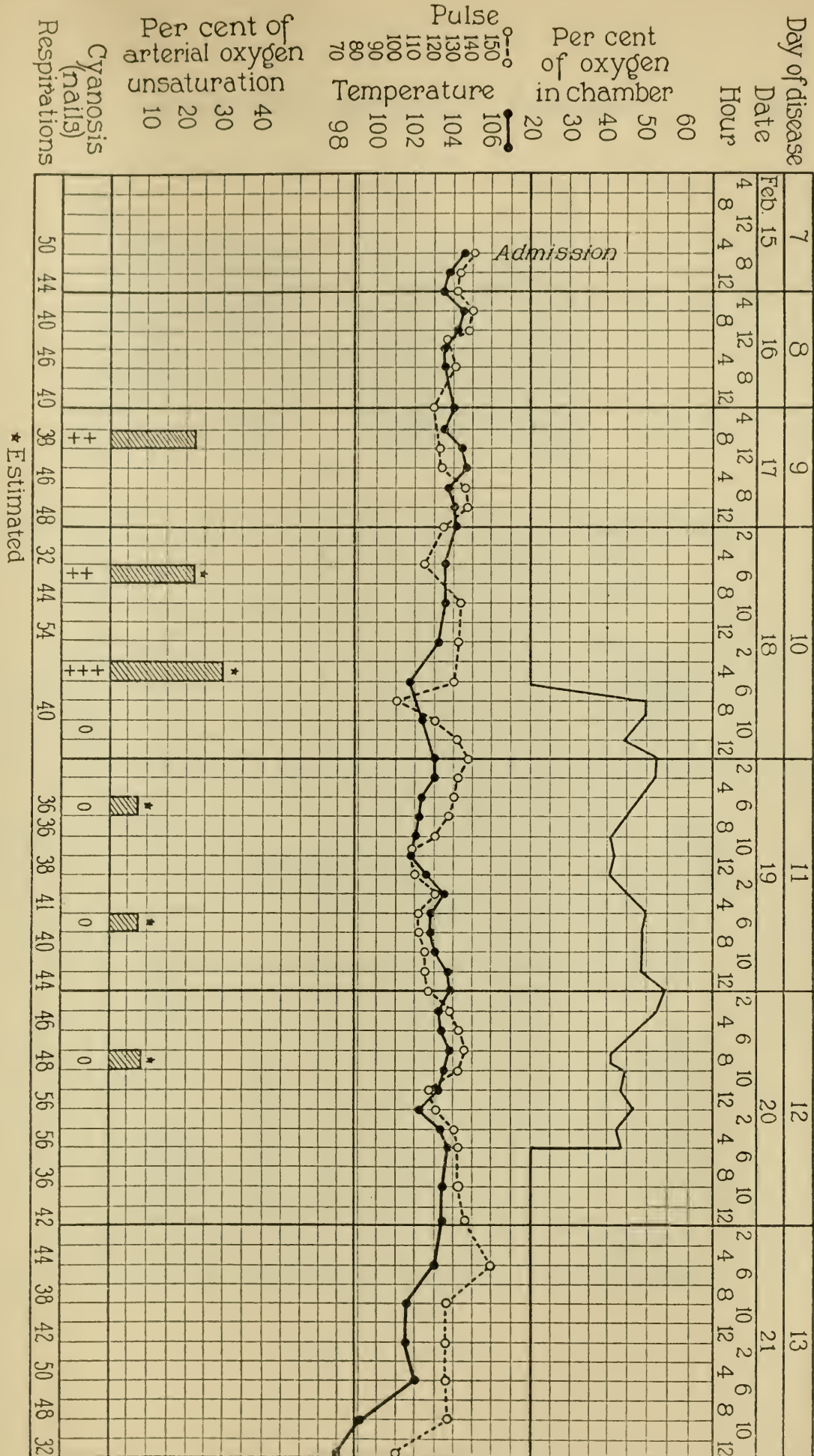
Mar. 2. Patient toxic. Cyanosis +. Consolidation over left upper lobe with scattered moist râles. 12 midnight. Patient is more toxic than this morning. Cyanosis the same (+).

Mar. 3, 6.30 a.m. Patient had a sudden attack of edema of the lungs; respirations rapid; pulse about 160, barely perceptible. Respirations labored. Coarse bubbling râles heard throughout the entire chest (Text-fig. 6). Patient deeply cyanotic (++++), cyanosis involving the entire hand, and face being very dusky; semicomatose. Condition grave. 9.30 a.m. Patient has been in the oxygen chamber since 6.30 a.m. His condition is still grave. Cyanosis (++) has cleared up in part; arterial unsaturation 10 per cent. There are still coarse râles throughout both lungs. 11.45 p.m. Patient's progress has been down hill despite the administration of 60 per cent of oxygen. Cyanosis (+++) distinctly increased. Lungs have rapidly filled up until everywhere there are large, coarse, bubbling râles. Pulse of poor quality during the day. 11.48 p.m. Died.

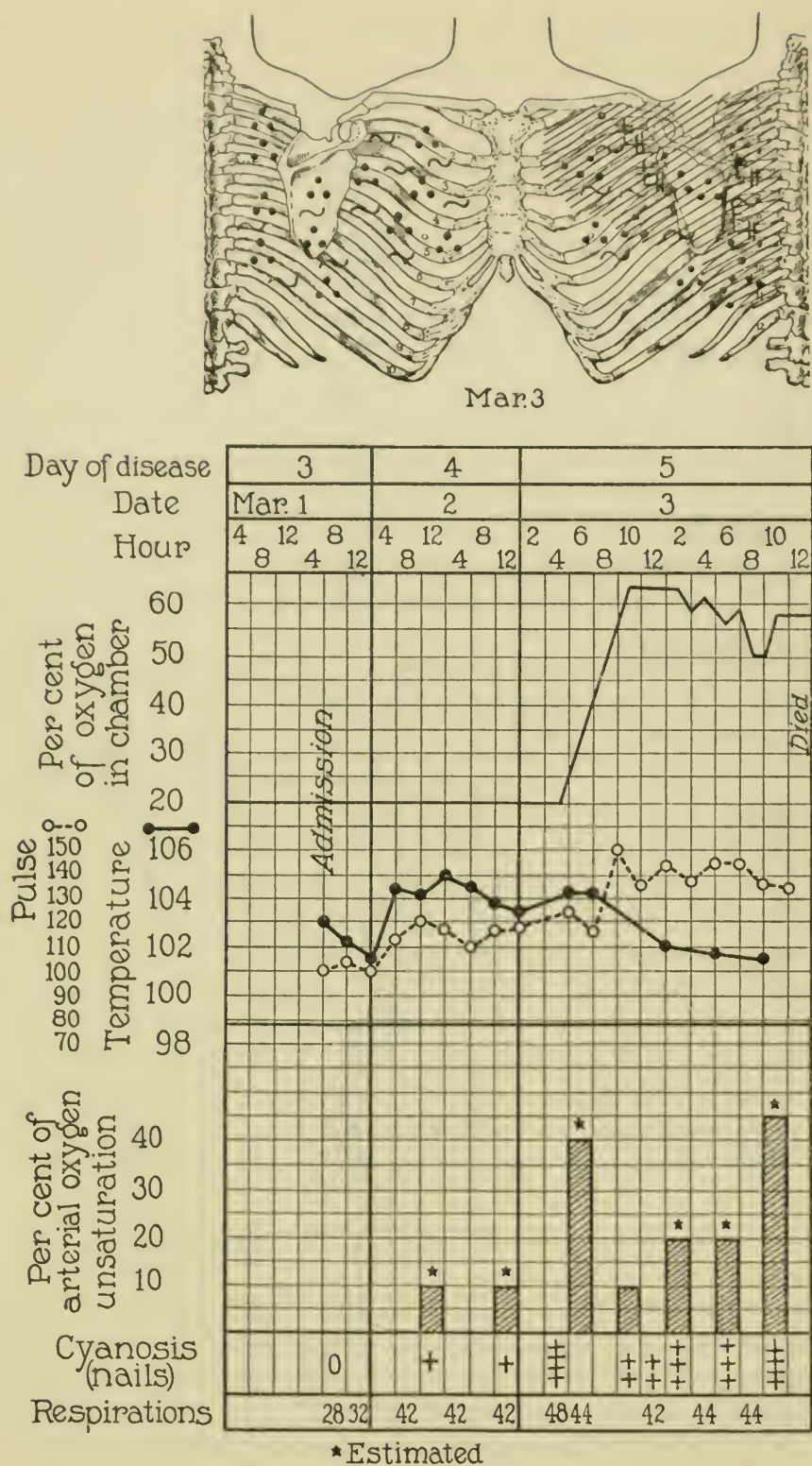
Bacteriological Examination.—Pneumococcus Type II from the sputum. Blood culture negative.

Autopsy.—Extensive tuberculosis involving the entire left lung, with cavity at the apex. At the right apex there was a small area of tuberculous infiltration. Both lungs showed extensive edema and many small patches of bronchopneumonia.

Discussion.—On account of the extensive tuberculosis the outlook in this case was, of course, hopeless. The sudden attack of edema of the lungs with the impairment of respiratory surface was accompanied by great cyanosis which

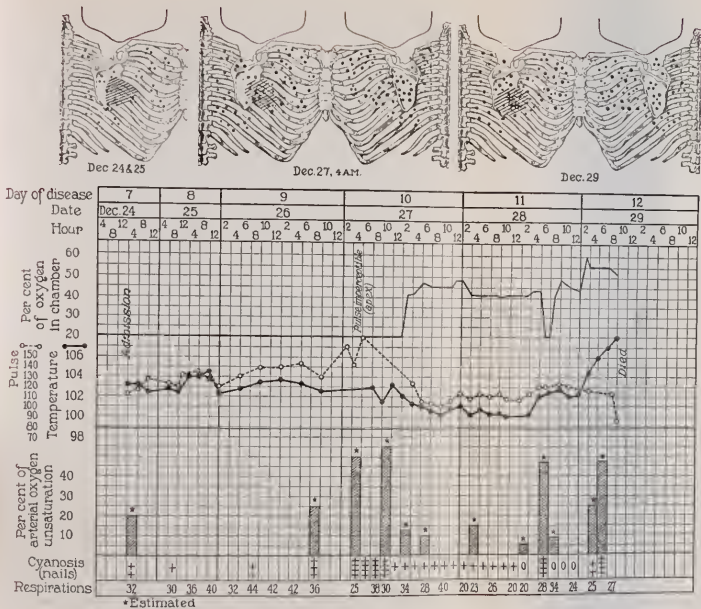


TEXT-FIG. 5. Case 5.



TEXT-FIG. 6. Case 6.

foldout



TEXT-FIG. 7. Case 7.

was temporarily relieved by the administration of high oxygen. In the face of the increasing edema the oxygen was subsequently without avail in relieving the cyanosis.

Case 7.—J. U., female, age 42 years. Admitted Dec. 24, 1920. Chief complaint pain in the back and cough.

Previous History.—The patient has been treated at the tuberculosis clinic at the Presbyterian Hospital for 5 or 6 years. All during this time she has had râles throughout both lungs. No definite consolidation. Tubercle bacilli have never been found in the sputum. Tentative diagnosis was tuberculosis.

Present Illness.—Onset Dec. 21, 1920, with severe backache, weakness, prostration, and cough. Temperature 103–104°F.

Physical Examination.—Patient restless, toxic. Temperature 103.6°; pulse 112; respirations 32. Lungs: Anteroposteriorly on both sides extensive medium sized, moist râles. Posteriorly at the fifth rib dullness and harsh breathing. Definite (++) cyanosis with estimated arterial unsaturation of 20 per cent.

Dec. 26. Patient's condition is graver. Pulse poor in quality. Respirations elevated. Cyanosis increased until it is about +++ with estimated arterial unsaturation of 20 to 25 per cent.

Dec. 27, 4 a.m. Patient's condition is critical. She is pulseless and deeply cyanotic (++++). Breathing gasping and difficult. Heart rate estimated at the apex to be 160. Condition looked almost hopeless. Respirations rapid and now slow, gasping and spasmodic. Patient comatose. Lungs: All over both sides extensive moist râles, fine, medium, and coarse (Text-fig. 7). 12 noon. Patient's condition worse this morning. Extreme cyanosis (++++). Estimated arterial unsaturation 50 to 60 per cent. Patient pulseless. Respirations still gasping. 12.10 p.m. Patient put in oxygen chamber with 50 per cent of oxygen. In a short time cyanosis had disappeared, respirations became more regular and rapid, going up to 36, and had lost the gasping quality. In a few hours pulse had returned to radial artery and had fallen to 100. Temperature also fell and patient was rational and presented the most remarkable contrast to her appearance this morning.

Dec. 28. The patient has been kept in the oxygen chamber with a percentage of 48 until last night at 12, when it was dropped to 40 per cent. Patient very restless from time to time. Pulse continues good. Respirations have dropped to 20, extremely labored, but deep and regular. Practically no cyanosis (+). Estimated arterial unsaturation 5 to 10 per cent. Lung signs about the same as those diagramed yesterday, except that râles are much less numerous. Over the upper portion of the right lower lobe there are definite signs of consolidation. 3 p.m. Patient's condition as far as temperature, pulse, and respirations were concerned was so good that the oxygen treatment was discontinued. Oxygen was allowed to leak out slowly and at 4 p.m. the doors were opened. 6.30 p.m. The patient was much worse; pulseless; breathing gasping in character; lungs showed more extensive râles all over; cyanosis ++++. Oxygen was again started and

rapidly run up to 40 per cent and later to 60 per cent. There was temporary relief of the condition, cyanosis disappearing completely.

Dec. 29. The increased oxygen undoubtedly tided her over the night. The improvement, however, did not last long. Patient soon became absolutely pulseless, extremities cold, comatose, had extreme jaundice, and died at 7 p.m.

Bacteriological Examination.—From the sputum was recovered a *Pneumococcus* Type IV. No tubercle bacilli found.

Discussion.—Patient had an old pulmonary history which suggested tuberculosis, although there was no definite proof of this. The case was remarkable in that with the definite spread of the initial consolidation an extensive involvement of the lungs was manifested by many fine and coarse râles on both sides. Patient became extremely anoxic and her condition quite serious. Administration of oxygen resulted in remarkable improvement in the condition which, however, was only temporary. No autopsy.

Case 8.—T. R., male, age 60 years. Admitted Jan. 4, 1921. Chief complaint pain in the right ear, headache, pain in the chest, cough, and expectoration of bloody sputum.

Present Illness.—Onset Jan. 3, with great prostration, and pain in the back and chest with severe cough.

Physical Examination.—Friction rub in right axilla; fine moist râles at right side anteriorly. Small area of dullness in posterior axillary line from the fifth to the seventh ribs with bronchial breathing. Left side clear. Cyanosis +.

Jan. 7. Patient quite toxic. Cyanosis +. Lung signs as diagramed in Text-fig. 8.

Jan. 9. Patient becoming sicker every hour. Cyanosis ++ all over. Right ear drum incised and pus obtained.

Jan. 10. Definite extension of the pulmonary process which now involves practically the entire right side. Patient is distinctly worse this morning. There is marked cyanosis (+++) and arterial unsaturation is 33 per cent. In spite of the almost hopeless outlook of this case, considering the extensive involvement and type of infection (*Pneumococcus* Type III) the patient is put in the oxygen chamber. 10 p.m. The effect of the oxygen was marked. Patient's color showed rapid improvement (cyanosis +) and toxic appearance greatly lessened. Blood culture + ; Type III *pneumococcus*, three colonies per cc. 11 p.m. Arterial unsaturation has decreased to 16 per cent. Cyanosis +.

Jan. 11. Condition about the same. Under the influence of oxygen he is a bright pink. Oxygen was discontinued for a short period this morning. Patient rapidly became much more cyanotic (++), with arterial unsaturation 20 to 25 per cent. Oxygen treatment recommenced. 11 p.m. Patient bright pink under 60 per cent of oxygen. Condition fair.

Jan. 12. Patient is much worse. Respirations are labored, pulse is very feeble, and he is markedly cyanotic (++++) despite 60 per cent of oxygen. Examination of the lungs shows extensive râles involving both lungs, with coarse bubbling râles all over. 8 a.m. Died.

35-2^a



Day of disease	
Date	
Hour	
Per cent of oxygen in chamber	60
	50
	40
	30
	20
Pulse	106
Temperature	104
	102
	100
	98
Per cent of arterial oxygen unsaturation	40
	30
	20
	10
Cyanosis (nails)	
Respirations	

Bacteriological Examination.—From the sputum and from the blood a Type III pneumococcus was obtained.

Discussion.—The extensive lung involvement and the type of infecting organism made the outlook in this case hopeless from the start. The initial effect of the oxygen was markedly to improve the patient's clinical condition as well as to relieve the cyanosis and anoxemia. When the oxygen was temporarily discontinued the patient relapsed into a toxic condition which was only temporarily relieved by a high percentage of oxygen.

DISCUSSION.

The frequent occurrence of anoxemia in lobar pneumonia is now definitely established. Analysis of arterial blood shows that even early in the course of the disease anoxemia is present in many cases and often may become a pronounced feature. As a rule, the anoxemia becomes greater as the disease progresses and invariably becomes marked 24 to 48 hours before death. Cyanosis is a regular accompaniment of anoxemia and varies directly with it.

The use of oxygen by inhalation is thus definitely indicated. By increasing the percentage of oxygen in the inhaled air its diffusion pressure is increased proportionately. More oxygen will pass through the lungs into the blood and the anoxemia will be diminished. This is strikingly shown in the cases reported. In several an arterial unsaturation of 35 to 50 per cent was quickly relieved and the blood became almost completely saturated. In other cases the anoxemia was partially relieved. In some instances, despite the administration of 50 to 60 per cent of oxygen the arterial unsaturation remained high. Cyanosis disappeared with the anoxemia and the bright pink color of the patients was in striking contrast to their previous purplish hue.

It is impossible to state definitely whether the removal of the anoxemia influenced the final outcome of the disease. The number of cases is far too small. The relief of anoxemia, however, appeared in most of the cases to be strikingly beneficial. No quantitative measure of this benefit can be given. But clinical evidence and impression, while not quantitative and always containing a personal element, are nevertheless valuable. All of the recovered cases presented grave prognoses, and several were in a critical condition. Case 1 was given two courses of oxygen. During the short interval out of the oxygen

chamber he became rapidly worse and it was the opinion of all who saw him then that his chances of recovery were slight. The immediate improvement under oxygen for the second time was striking. Case 2 showed the same improvement on the first administration of oxygen. In a short time after removal from the oxygen chamber the patient was in great distress. The cyanosis was + + +, the arterial unsaturation 35 per cent, the breathing difficult and labored. There was beginning jaundice which later became intense. There was every indication that the patient was dying. The change in the general condition of the patient shortly after the second administration of oxygen was as remarkable as in the previous case. Case 7 gave a convincing demonstration of the distinctly beneficial action of oxygen in an anoxic pneumonia patient. The patient, obviously dying, was pulseless, the heart rate was 160 to 170, there was deep cyanosis, the extremities were cold, her respirations gasping, and she was comatose. The lungs were filled everywhere with coarse, bubbling râles. Yet within a short time after the oxygen treatment was begun the condition had changed completely. Then the patient was bright pink, the pulse had returned to the radial artery, and it was strong (120), respirations were quieter, and she was mentally alert. The fact that the patient died finally makes the demonstration of the action of oxygen more convincing in as far as the immediate relief of grave symptoms is concerned. The remaining cases likewise showed in general the same changes in the clinical picture following oxygen.

These patients are, then, examples of critical cases of pneumonia which sooner or later present a picture of severe toxemia upon which is added a marked disturbance of the oxygen transfer from lungs to tissues. They are thus struggling against infection with a marked handicap, and our experience has shown that these anoxic patients do not do well. In the last analysis recovery will depend upon the specific resistances to the infection which the patient can muster, but while oxygen can in no sense be specific, the above cases strongly indicate that marked improvement follows its administration and presumably life was prolonged. In five of the eight cases recovery ensued. Ordinarily the recovery of these cases was extremely doubtful.

There were three fatal cases. Of these, one (Case 6) had, as shown at autopsy, extensive tuberculosis upon which was superimposed a fresh pneumonia. Case 7 had been treated at the Presbyterian Hospital for years for a chronic bronchitis and was a suspected tubercular case although no definite proof of this was found. The third fatal case (No. 8) was a man of 60. The type of infecting organism (Type III pneumococcus) present in the blood and lungs is known to be highly virulent and the mortality in cases with a positive blood culture is close to 100 per cent.

On the whole, the series here reported, though small, is encouraging. The relief of anoxemia by oxygen appears to be beneficial.

It would perhaps be better to commence oxygen treatment as soon as anoxemia is observed. Any of its harmful effects could then be prevented.

In order that oxygen treatment in pneumonia should be of benefit it must be given over a long period of time. It is futile to give oxygen haphazardly at infrequent intervals, and by methods which are wasteful. Moreover, the percentage of oxygen which reaches the lungs should be approximately known and controlled. The inhalation for a long time of 100 per cent of oxygen is known to be injurious (Smith, 1899), and unless oxygen is given by properly controlled methods, it is possible that the alveolar air may be almost pure oxygen. An oxygen chamber is the method of choice. The patient suffers no added discomfort, and can receive nursing and medical attention as if in the open ward. Moreover, a definite percentage of oxygen can be given. Accurate observation of the course of the disease can be made under known conditions.

Other devices may be used. The Haldane (1917) mask is efficacious and economical. Hill (1921) has devised a bed tent for use in oxygen therapy. Meltzer (1917) used his insufflation method in pneumonia and reported striking results. With these and other devices which may be easily constructed the further study of the action of oxygen as a therapeutic measure can be made. The essential features of such devices should be ease and economy of operation, adequate removal of carbon dioxide, moisture, and heat, and comfort for the patient with as little interference with the medical and nursing care as possible. Most important of all, the device must

enable oxygen to be given over a long period of time and the percentage of oxygen must be easily controlled.

Mode of Production of Anoxemia in Pneumonia.

A study of cyanosis and anoxemia in relation to lung signs in a large series of cases has shown that the extent of the cyanosis depends upon the amount and kind of involvement. This becomes evident when careful daily plotting of lung signs is done together with observations of the anoxemia from the arterial unsaturation and cyanosis. A few typical examples are given in the lung charts accompanying the case reports. As a rule when the pneumonic process is confined to one area, as in the typical lobar pneumonia variety, there is little or no cyanosis. The physical signs, *viz.* dullness, bronchial breathing, and a few fine râles, are then confined to the area involved. Kline and Winternitz (1915) and later Gross (1919) have shown that in this type of consolidation there is little or no circulation of blood to the consolidated lung, and therefore little or no admixture of non-aerated with aerated blood occurs. When, however, there is a spread of the pneumonic process, and there occur scattered and secondary areas of bronchopneumonia, the blood circulation in these areas is intact. Moreover, there is a more or less widespread bronchitis with considerable peribronchial pneumonia and edema. The lung signs are changed. In addition to the original area of dullness and bronchial breathing, there are other areas of impaired resonance and harsh breathing. Also râles are heard over a considerable part of the lung, sometimes on one side, often on both. These râles are medium sized or coarse, moist râles, and indicate a considerable involvement of the lungs with edema, consolidation, and pneumonic infiltration. Many of the alveoli are thus functionless, others are coated with exudate, bronchioles are wholly or partially occluded, and the efficient ventilation of the lungs is greatly hindered. Anoxemia results.

A study of the lung signs in the case reports shows that anoxemia is proportional to the extent of respiratory surface involved. In Case 1 on March 25 there was a complete consolidation of the right lower lobe. There was no cyanosis. On March 28 and 29 there was a definite spread of the initial process and many fine râles were present on the opposite side. The anoxemia was now 37 per cent with a

+++ cyanosis. On March 30 the process was still more extensive, coarse moist râles were heard on both sides, and without oxygen the arterial unsaturation rose to 30 to 40 per cent (estimated) with a ++++ cyanosis. The moist râles persisted for a number of days and during that time even under increased oxygen some anoxemia was present.

Case 2 more strikingly illustrates this. On February 4 there were signs of consolidation over the right lower lobe only, with no cyanosis. On February 7 the consolidation had extended and a marked impairment of the respiratory surface was indicated by the presence of numerous coarse râles on both sides posteriorly. There were now a ++ cyanosis and 22 per cent of arterial unsaturation. On February 9, when oxygen was withdrawn, anoxemia reappeared (35 per cent of arterial unsaturation). There were still many coarse râles on both sides. On February 11 the râles had disappeared, leaving only the initial area of frank consolidation. The cyanosis was +, the arterial unsaturation normal.

In Case 3, on April 29 and 30, with signs of consolidation over the entire left back, the anoxemia was considerable (cyanosis ++; arterial unsaturation 20 per cent). On May 1 the involvement was more extensive, with coarse râles all over the left side. The cyanosis (+++) and arterial unsaturation (25 per cent) were definitely increased.

In Case 6, on March 1 there was no cyanosis. The lung signs (not charted) were dullness and bronchial breathing over the left upper lobe with fine moist râles. On March 3 the signs had spread, involving the entire left lung. There were numerous coarse râles on both sides. The cyanosis was ++++.

In Case 7, with an increase in the extent of pulmonary involvement as indicated by an increase in the number of râles, the anoxemia definitely increased.

In Case 8, on January 7 there was slight anoxemia (cyanosis +) with a small area of consolidation and scattered râles over the right upper lobe. On January 10 the entire upper and middle lobes showed dullness and bronchial breathing. There were coarse râles over the entire right side. Anoxemia was marked (arterial unsaturation 37 per cent; cyanosis +++).

These protocols show definitely that a spread of the pneumonitis, as indicated by dullness, bronchial or harsh breathing, and especially the presence of edema as indicated by râles, is associated with increase in the anoxemia. Whether the amount of pneumonitis, bronchitis, and edema is the sole factor in the production of anoxemia is at present difficult to state.

Haldane, Meakins, and Priestley (1919) studied rapid and shallow breathing in normal subjects. In some cases they produced periodic breathing, but only in one experiment was cyanosis observed. In this case the subject was breathing at the rate of 156 per minute, the tidal air being 159 cc. In other experiments the respiratory rate varied from 69 to 150 per minute and the tidal air was 69 to 161 cc. No cyanosis was reported in these cases even when the tidal air was as low as 69 cc. No blood analyses were done. Haldane, Meakins, and Priestley conclude: "that the shallow breathing causes uneven ventilation of the lungs and this in turn produces anoxemia and consequently periodic respiration and other symptoms." Meakins and Davies (1920) repeated this work with analysis of the arterial blood. In the one experiment reported they found that a change in the tidal air from 586 to 315 cc. produced a diminution of the arterial saturation of 2.6 per cent. On the basis of this and the previous experiments with Haldane and Priestley, Meakins (1920, *b*) concludes that rapid and shallow breathing produces anoxemia in pneumonia, and he reports four cases in which the tidal air and extent of cyanosis were studied. In these cases he found that with a decrease in the tidal air there was an increase in the cyanosis. No protocols of lung signs are given. Later (1921) he states that: "the degree of pulmonary consolidation is of less importance than the type of interference with respiratory function" in the production of anoxemia.

The extremely rapid and shallow type of breathing studied by Haldane, Meakins, and Priestley is, of course, quite outside of any observed range in health or disease. In studies of many cases of pneumonia I have rarely observed a tidal air below 250 cc. Moreover, cyanosis was apparently a rare occurrence even with this extreme type of shallow breathing. In the experiment of Meakins and Davies (1920) the tidal air was cut in half. This is much more like the condition met with in pneumonia. The diminution of the arterial saturation was slight, only 2.6 per cent, or about 0.5 volume per cent in average blood. This is in striking contrast to the 20 to 40 per cent of arterial unsaturation frequently observed in pneumonia patients whose tidal air ranges between 250 and 350 cc. While it

is possible that the type of breathing may be responsible in some measure for the production of anoxemia, it is difficult with the evidence at hand to see how it can be the sole or even the most important cause of the great anoxemia often associated with pneumonia.

SUMMARY.

1. The use of an oxygen chamber in the treatment of pneumonia patients makes it possible to administer this gas for long periods of time under exactly known conditions. The medical and nursing care of the patient is greatly facilitated.

2. Prolonged inhalation of oxygen varying from 40 to 60 per cent appears to be without harm.

3. Oxygen administered to intensely anoxic patients almost immediately clears up this anoxemia. Cyanosis disappears with the anoxemia.

4. The removal of patients from the high oxygen while they are still sick and while examination shows that there are still extensive edema and infiltration of the lung results in a return of the intense anoxemia.

5. It is sometimes impossible to clear up the anoxemia, even when as high as 60 per cent of oxygen is given, especially when there are considerable edema and infiltration of the lungs.

6. Five cases in which the prognosis was grave recovered. Three cases, one of tuberculosis, one with a *Pneumococcus* Type III infection, and a third with a pneumonia superimposed on a chronic pulmonary condition, died.

7. In all cases there appeared to be an improvement in the patient's condition. In one case, particularly, with an intense degree of anoxemia, the patient became moribund and pulseless. Following the administration of 60 per cent of oxygen there was a lowering of the heart rate from 160 to 120, the return of the pulse to the radial artery, the color became bright pink, and there was a remarkable change in the clinical condition.

8. The anoxemia of pneumonia is due, in large measure, to an impairment of the respiratory surface of the lungs. The greater the lung involvement the greater the anoxemia. Especially is this so

when the pneumonic process extends throughout the lungs so that there are many patches of bronchopneumonia, with accompanying bronchitis and edema, as evidenced by the presence of râles throughout the lungs.

9. Rapid and shallow breathing of the degree observed in pneumonia is, as far as present evidence shows, of less importance in the production of anoxemia.

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AN ORGANISM RESEMBLING BACILLUS ACTINOIDES ISOLATED FROM PNEUMONIC LUNGS OF WHITE RATS.

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PLATE 10.

(Received for publication, October 20, 1921.)

During the past few years several have isolated organisms from rats suffering from pneumonia. Mitchell¹ cultivated from the lungs of rats during an outbreak of pneumonia a granular Gram-positive bacillus. Klein² had previously encountered a similar bacillus in two cases of rat pneumonia. This organism he named *Bacillus muris*. *Bacillus muris* grew well on the ordinary media. It produced acid in dextrose broth or dextrose serum water. Cultures injected into the pleural cavity of rats and mice set up a pleuritis and pneumonia with fatal termination. Subcutaneous injection into rats and guinea pigs produced local abscesses. Tunnicliff³ succeeded in cultivating a delicately growing streptothrix from the lungs of twenty rats. This organism failed to grow on ordinary media but good growths were obtained on media which contained ascitic fluid. The streptothrix was Gram-negative. It was not acid-fast. Branching was observed only in young cultures. It was described as slender, straight or wavy filaments which soon tended to fragment. The culture was pathogenic for rats when injected into the pleural cavity. Recently Hoskins and Stout⁴ have obtained *Bacillus bronchisepticus* from the upper respiratory tract and lungs of rats suffering with respiratory disease. From descriptions encountered in the literature one would assume that several types of lung disease exist in the rat.

The writer undertook the study of rat pneumonia and succeeded in obtaining from eleven cases organisms closely resembling those isolated by Theobald Smith^{5,6} from the pneumonic lungs of calves.

¹ Mitchell, O. W. H., *J. Infect. Dis.*, 1912, x, 17.

² Klein, E., *Centr. Bakt., 1te Abt., Orig.*, 1903, xxxiii, 488.

³ Tunnicliff, R., *J. Infect. Dis.*, 1916, xix, 767.

⁴ Hoskins, H. P., and Stout, A. L., *J. Lab. and Clin. Med.*, 1919-20, v, 307.

⁵ Smith, T., *J. Exp. Med.*, 1918, xxviii, 333.

⁶ Smith, T., *J. Exp. Med.*, 1921, xxxiii, 441.

He has named the calf organism *Bacillus actinoides*, *n. sp.* (Smith, 1918). Certain well defined morphological characters which he described render its recognition a relatively simple matter. His cultures when injected into the tracheæ of calves produced circumscribed necroses of the lung. Subcutaneous injections gave rise to large indurations ending in necrosis.

It must not be assumed that the *Bacillus actinoides*-like cultures were obtained from every rat examined. In a few instances *Bacillus bronchisepticus* was isolated. Cocci of various kinds were frequently found. Streptothrices were observed in a few tubes. In many instances growth could not be obtained.

Cultivation from the lungs of rats presents certain difficulties. Involved lobes are usually shrunken and afford very little material. The disease is a chronic one, offering excellent opportunity for invasion with more rapidly growing bacteria. Experimental work with organisms isolated is equally difficult. Practically all rats which have come under my observation after reaching maturity have spontaneously developed lung disease.

Nothing definite can be said at this time regarding the real relationship of the organism about to be described to certain rat pneumonias. On the other hand, the remarkable similarity which exists between Smith's calf cultures and those I have obtained from the white rat is worthy of emphasis. The pneumonic processes of the rat disease resemble in certain respects those described by Theobald Smith in calves.

Method of Cultivation.

The rat is chloroformed. The skin over the thorax is shaved and disinfected. After the musculature has been exposed it is seared. Portions of the ribs and sternum are removed with sterile instruments. In the main the culture methods described by Smith have been employed. Small pieces from the involved lobes are placed in tubes containing the solid media. The pieces are pushed down the tube and over the surface of the slant and lodged in the liquid at the bottom. The tube is then sealed with sealing wax. Slanted coagulated horse serum to which 0.5 cc. of sterile calf serum water has been added to the condensation fluid has on the whole given the best results. Growth

has also been obtained on slanted veal infusion agar to which either calf serum water or defibrinated horse blood has been added to the original condensation fluid. Emphasis is placed on the thorough sealing of the tubes.

Growth Characters.

3 or 4 days after inoculation with bits of lung tissue, tiny scintillating particles may appear within the condensation fluid and calf serum water. As a rule colonies on the slant are not visible to the unaided eye. With a lens, however, very tiny white colonies are observed. The tiny particles gradually become larger and finally appear as irregular gray flocculi suspended in the liquid. In certain cultures a distinct cream-like surface layer in the condensation fluid has been observed in old cultures. The surface colonies increase in size and become round, white, smooth protrusions (Fig. 1). On the agar media the growth is more delicate. Here the condensation fluid becomes slightly turbid and either a very delicate translucent film or discrete colonies appear on the slant. The colonies are very small, usually under 0.5 mm. in diameter. They are round, translucent, and raised. The growth in the fluid at the bottom tends to become viscid. After the organism becomes established on the artificial media it is possible to study its morphological characters in detail. The coagulated serum cultures are in many respects most interesting.

If a 48 hour subculture on coagulated serum be examined with the naked eye little except barely visible scintillating particles in the condensation fluid attracts attention. Hanging drop or stained preparations from the liquid show that the particles are made up of aggregates of fairly long, slender, non-motile rods. They appear to be embedded in clear (unstainable) areas (Fig. 3). Chains of four or five individuals are not infrequent. Preparations from the slant reveal similar organisms. At the end of 4 days the particles have increased in size and appear as tiny gray flocculi. Microscopically they contain many interwoven filaments. A few may protrude from the edges of the mass. The organisms appear granular. About the borders of certain clumps one may encounter a few clear, pear-shaped bodies. Preparations from the slant reveal a few rod-like elements and great numbers of rounded refringent bodies in clumps. When

the condensation water is examined after 7 days, the flocculi are larger. They now appear as mulberry masses of pear, slipper, or club shape, or rounded, clear globules. Scattered throughout this substance are small groups of crystals. The structure of the mass is best shown by placing a drop of the liquid on a slide, with gentle pressure the club-like elements can be made to flow. Fig. 2 illustrates the appearance of a well developed flocculus in the condensation water. The details of the edge of a similar flocculus from an older culture show the characteristic clubs and a few crystals (Fig. 4). The resemblance between these club-like masses and preparations of the ray fungus from actinomycotic lesions is striking. Stained preparations add little of value. The capsular substance does not stain with any of the ordinary bacterial stains. As a rule a few very faintly stained filaments and fragments are visible in the center of the flocculus (Fig. 5). Theobald Smith⁷ in a recent article has described in detail the formation of the capsular material. The clubs appear first as terminations of the filaments. He points out that the original substance resembles myelin, but that fat granules make their appearance as the culture becomes older and continue to develop at the expense of the myelin-like substance. On the surface of the slant in 7 day cultures the clubs are usually much smaller. Many of the long rods have fragmented so that it is possible to observe masses of very short refringent forms and long filaments. Both types are illustrated in Fig. 6. The shorter forms stain poorly, the long filaments are more deeply colored. Branching has not been observed.

On the blood agar media a much different process is revealed. Here the rods in the young cultures (48 hours) vary considerably in length. Rods from 5 to 10 microns are observed. They are usually straight or slightly curved and often beaded. The ends may be tapered or the bacillus may terminate in a distinct swelling. Constrictions of the body of the organism are not uncommon. Many of these forms have been photographed in Fig. 7. After 5 to 7 days the rod forms have disappeared and hanging drop or wet mount preparations reveal only refringent coccoid elements (Fig. 8). It has been stated that rods and filaments with swollen ends are noted during the first 48 hours of incubation. The swellings become more marked

⁷ Smith, T., *J. Exp. Med.*, 1921, xxxiv, 593.

during the 3rd and 4th days. The body of the organism shrinks and finally all that remains is the refringent spore-like mass. These masses when introduced into fresh media will reproduce the characteristic pleomorphic rods which undergo the changes described.

Although such organisms have been observed in the cultures from eleven cases, it is only with difficulty that they can be maintained. In several instances *Bacillus actinoides* was associated with a delicately growing streptococcus. It was possible to carry along these mixed cultures in serum media for several generations, ultimately only the streptococci survived. These mixed cultures made the microscopic study exceedingly difficult. It was only after a few pure cultures of the rods were obtained that the nature of the growth process became clear. Thus far it has not been possible to transfer cultures from the coagulated serum media to blood agar or calf serum water agar. Attempts to transfer from the agar to serum media have also failed.

The organism when grown on agar stains easily in young cultures with methylene blue or carbolfuchsin. It is Gram-negative. Preparations from the serum media are more difficult to stain.

The pathogenic properties of certain strains have been tested in a few instances. White rats injected with cultures beneath the skin suffered no ill effects. Several experiments were made in which the culture was introduced into the trachea of rats. Unfortunately the controls developed a spontaneous pneumonia so that nothing definite could be determined. A calf injected beneath the skin of the neck with a typical rat culture developed only a mild local reaction.

SUMMARY.

An organism obtained from the pneumonic lungs of eleven white rats is described. It has been maintained in pure culture in a number of instances. In young cultures the organism appears as a long slender bacillus. In older cultures on coagulated serum media characteristic club-like capsular material is formed. On blood agar characteristic swellings appear at one or both ends of the rod. These become more refringent, and the body of the organism begins to shrink. Finally only rounded spore-like refringent bodies are found. The organism is Gram-negative.

EXPLANATION OF PLATE 10.

FIG. 1. A culture, 3 months old, from the ventral lobe of Rat 1, Experiment 5, on coagulated horse serum. The surface growth is characteristic, a few larger flocculi are visible suspended in the condensation water. Many have sunken to the bottom. Natural size.

FIG. 2. A large flocculus and several smaller ones from the condensation fluid of a 7 day serum culture. $\times 25$.

FIG. 3. Stained preparation from the condensation fluid of a 48 hour coagulated horse serum culture, Rat 53. Note the aggregates of rods in the clear spaces. Stain is dilute carbol-fuchsin. $\times 1,000$.

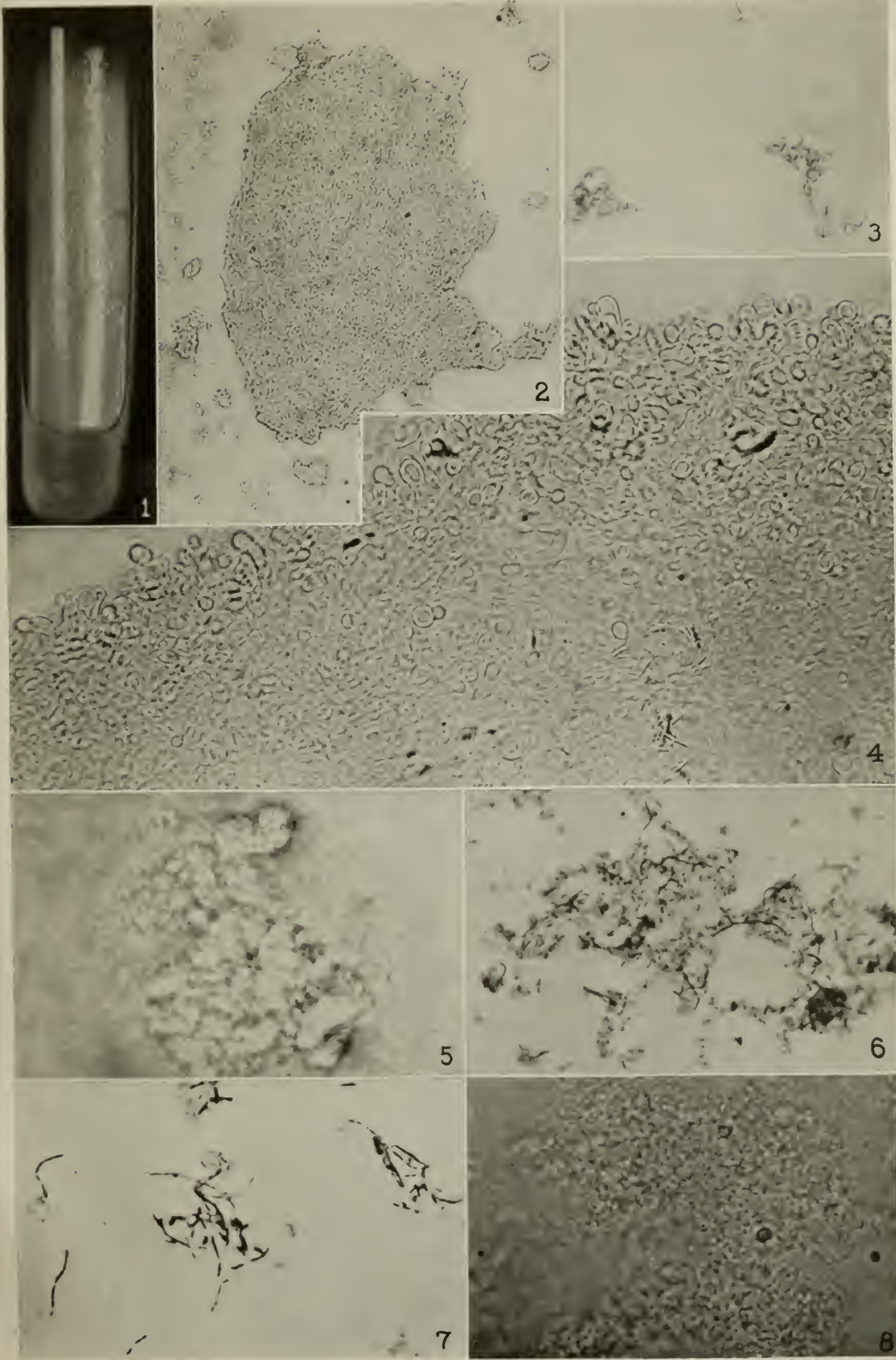
FIG. 4. The edge of a flocculus similar to that shown in Fig. 2. The preparation is from the liquid of a 2 weeks old culture on coagulated serum. The clubs in several planes appear as protruding pear-shaped, rounded, or oval, clear bodies. A few needle-like crystals are present. $\times 550$.

FIG. 5. A flocculus from the condensation fluid of a 7 day serum culture stained with methylene blue for 12 hours. A few faintly stained filaments and fine granules are visible. The capsular substance is unstained. $\times 1,000$.

FIG. 6. Stained preparation from the slant of a 7 day culture on coagulated serum. Note the long filamentous forms which stain well and the fragments which stain only faintly. One rod appears to have a branch leaving the main filament at an angle of about 45° . The microscope reveals that these organisms are entirely separate. The stain is dilute carbol-fuchsin. $\times 1,000$.

FIG. 7. A stained preparation from the slant surface and condensation fluid of a 48 hour blood agar culture, Rat 42. Various forms are clearly illustrated. $\times 1,000$.

FIG. 8. An unstained wet mount from the lower portion of the slant and condensation fluid of a 12 day blood agar culture, Rat 42, showing the refractile spore-like bodies. $\times 650$.



366²-

(Jones: Organism from pneumonic lungs of rats.)

A THREE MONTHS OLD STRAIN OF EPITHELIUM.

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PLATES 11 TO 14.

(Received for publication, October 20, 1921.)

The purpose of the experiments described in this paper was to obtain a pure strain of epithelium and to keep it permanently *in vitro*, as has been done with connective tissue. Epithelium taken from chickens, dogs, human beings, etc., has already been grown *in vitro* for a short period of time and its characteristic growth described.¹ Ruth,² Oppel,³ Holmes,⁴ and Uhlenhuth⁵ have cultivated epithelium from cold-blooded animals. Carrel⁶ attempted several times to obtain a permanent strain of epithelium from the skin of chick embryos, but after 2 or 3 weeks the cultures were invaded by fibroblasts and the epithelial cells progressively disappeared. The transformation of the cultures of epithelium into cultures of connective tissue was probably not caused by dedifferentiation of epithelial cells into fibroblasts, as has been stated by Champy,⁷ but by contamination of the cultures by fibroblasts. The fibroblastic contamination was due to the difficulty of obtaining for culture epithelium completely free from connective tissue cells. Ebeling⁸ succeeded in keeping pure cultures of corneal epithelium which spontaneously ceased to grow after a few weeks. Although the cultivation of epithelium appeared to be more difficult than that of connective tissue, it seemed probable

¹ Carrel, A., and Burrows, M. T., *J. Am. Med. Assn.*, 1910, lv, 1379; *J. Exp. Med.*, 1911, xiii, 416.

² Ruth, E. S., *J. Exp. Med.*, 1911, xiii, 422.

³ Oppel, A., *Anat. Anz.*, 1913-14, xlv, 173.

⁴ Holmes, S. J., *Univ. California Pub. Zool.*, 1913, xi, 155.

⁵ Uhlenhuth, E., *J. Exp. Med.*, 1914, xx, 614.

⁶ Carrel, A., unpublished experiments.

⁷ Champy, C., *Bibliog. Anat.*, 1913, xxxiii, 184.

⁸ Ebeling, A. H., unpublished experiments.

that if epithelial cells could be obtained free from connective tissue cells, they could be kept in pure culture indefinitely. The strain of epithelium described in this article was isolated from chick embryo eyes. Fragments of tissues were taken from different parts of the eye, tapetum layer, cornea, and lens, in the hope of obtaining pure epithelium. Only a few cultures from the lens produced pure epithelium, and by repeating the explantation of different tissues from the eye, it appeared that only a certain kind of supposed lens cultures produced a pure outgrowth of epithelium, namely, the peripheral portion which had a little brim of iris epithelium attached. The lens tissue itself did not grow at all, but the little brim of iris which sticks to the lens when it is enucleated grew out apparently as pure epithelium. After 3 months cultivation *in vitro* it still looks as pure as when it was observed on the 1st day. Now and then a few elongated, spindle-shaped cells resemble fibroblasts, but under high magnification they are easily recognized as epithelial cells on account of their similarity to the other cells in the culture, the pigment which the iris epithelium contains, and their characteristic way of growing in close contact with the neighboring cells.

I.

EXPERIMENTAL.

1. *Preparation of the Tissue.*—Epithelial tissue for cultures is obtained from the eye by taking out the lens with a cataract knife. In most cases a very thin black brim of the iris adheres spontaneously to the peripheral part of the lens. The lens may be cut in three or four parts and placed in the ordinary plasma-tissue juice culture medium. The lens itself does not grow, but sometimes the outgrowth of epithelium from the iris can be observed after 48 hours, although frequently not until after several passages. If any fibroblasts grow out in the same culture, which will be noticed readily, there is little hope of obtaining pure epithelium from it. If parts of the iris are allowed intentionally to remain on the lens, the growth will be largely composed of fibroblasts. The extirpation is carried out as if only the lens is to be removed, and only that part of the iris which spontaneously remains is suitable for obtaining a pure epithelial culture.

2. *Nature of the Medium.*—The fact that the epithelial cells liquefied the plasma clot much more extensively than fibroblasts suggested that if the cells were cultivated under conditions in which a kind of liquefaction has already taken place, a more extensive and uniform growth would result. An attempt was then made to place the tissue on the free surface of the already coagulated plasma (one volume of plasma and one volume of embryonic juice), and to cover it with a small drop of embryonic tissue juice. This method of cultivation appeared to be the most satisfactory. As a rule, the growth took place in one delicate, continuous layer on the surface of the clot.

3. *Preparation of the Cultures.*—Equal volumes of plasma and embryonic tissue juice are mixed on the cover-slip and allowed to coagulate. After coagulation commences, which can easily be ascertained with the point of the knife, the tissue is placed on the free surface of the nascent clot. If this precaution is not taken, the tissue will float in the tissue juice which is afterward added, and no growth will occur. It is important to place the tissue on the clot at the proper moment; if too early, the tissue will be embedded, and if too late, it will not adhere. Fixation is the desired object. After the fragment has been properly placed on the clot, a small drop of embryonic juice is spread evenly, in a thin layer over the tissue and beyond its margin, to afford a moist surface for proliferation. When transferring the culture, the excess fluid is removed by means of a piece of sterile filter paper, and the culture cut in the usual way. Because of the retraction of the tissue, it is most important to extirpate the tissue in such a way that no part of the plasma clot remains in the periphery of the excised culture. Otherwise, the cells will be embedded in old clot and further growth will be prevented.

It is advisable to embed the cells in the medium during the first few passages. If connective tissue cells should be present, they are much more easily distinguished, because of their characteristic appearance when they grow in the middle of the clot. When no fibroblasts can be detected, the cultures are allowed to grow on the free surface of the clot, covered only by a small drop of tissue juice.

II.

RESULTS.

The outgrowth of new cells appears in a fine mosaic structure. The size of the epithelial layer of conglomerated cells depends very much on the consistency of the media. When cultivated on the free surface of the plasma clot, the new growth appears as a continuous sheet of cells in pavement formation (Fig. 1). When cultivated in the middle of the clot, smaller and larger peninsulas of cells grow out, and sometimes a few single cells.

For some while there has been a discussion as to the reason for the disappearance of epithelium when cultivated *in vitro*. This fact has been brought out in connection with an assumption by Champy⁷ that a dedifferentiation of the epithelial cells to the type of fibroblasts takes place *in vitro*. This does not seem to be correct, and is more an apparent phenomenon. Several investigators, among them Uhlenhuth,⁹ have shown that the shape of the cells cultivated *in vitro* depends upon the structure and consistency of the culture medium; in other words, on purely mechanical facts. By taking the precaution of cultivation under the same mechanical conditions, namely on a moist surface of plasma clot, as herein described, the cultures present as typical epithelial characteristics after 3 months cultivation as they did on the 1st day. Whenever it is desired, the epithelial cultures can be embedded in the solid plasma clot and the supposed dedifferentiated epithelial cells can be reconstructed; and repeated cultivation on the surface brings the fusiform cells back to the polygonal type of epithelium. It is purely a matter of mechanical conditions. In the periphery of ordinary, well grown epithelial cultures, made on the surface of the clot, some more or less elongated, spindle-shaped cells appear. These are found mostly when the outlines of the growth reach the outlines of the moist surface; *i.e.*, the border of the area which is covered by the tissue juice. Then they embed themselves in the solid clot and become elongated because of the dense medium. This same phenomenon has been described by Uhlenhuth⁵ in his experiment on epithelial cultures from frog skin. He seems to have found just the reverse

⁹ Uhlenhuth, E., *J. Exp. Med.*, 1915, xxii, 76.

of the observation stated above; namely, that the elongated, fusiform, epithelial cells grow out only in the very "soft medium," and in compact sheets when cultivated in a "firm medium." This may be explained in the following way: when the culture medium is made as soft as in Uhlenhuth's⁹ experiments, but not quite fluid, the fibrin fibrillæ are so sparsely present that the few cells which grow out stick to them as the only support, and become spindle-shaped for that reason. The same phenomenon occurs when tissue is cultivated in a fluid medium in which cotton threads¹⁰ or a spider net (Harrison¹¹) is used for framework. In some cases, when cultures were made in the firm clot, it may be that the tissue cells found their support dense and homogeneous. In others, the transplanted fragment of tissue may have been more or less near the surface of the clot and, for that reason, the cells may have grown out on the surface in continuous layers.

The epithelial strain now under cultivation is very easily multiplied. The strain took its origin from five cultures, and in the elapsed time they have been increased to between 50 and 60, though the speed of the growth seems to be slower than that of fibroblasts. The ability to proliferate seems also to have increased recently; whether this is due to an improved technique in making the cultures or whether the cells have become better adapted to the life *in vitro* is difficult to say at present. It is noteworthy that the cultures made from fresh tissue are often continued for several passages before the epithelium begins to grow. A similar stage of latency has been observed by Uhlenhuth⁹ in epithelial cultures.

The epithelial cells in cultures cause a liquefaction of the plasma clot to a higher extent than do those of connective tissue cells, and the epithelial tissue itself when excised from the culture has a peculiar mucous character and retracts easily, as does a drop of mucus. The most characteristic feature about the growth of epithelium is that the cells keep close together and grow in pavement formation. Numerous mitoses are found in the cultures and different stages of amitotic cell division (Figs. 2, 3, and 5). Most of the investigators who have cultivated epithelial cells *in vitro* state that they did not

¹⁰ Fischer, A., unpublished experiments.

¹¹ Harrison, R. G., *J. Exp. Zool.*, 1914, xvii, 521.

observe any mitosis. After cell division, the new cells do not seem to move very far from the point where the division took place. The photographs show such an ability to form a continuous layer (Figs. 1 to 4). Very often in looking at the cultures, one is reminded of a cross-section of epithelial glandular tissue; that is, not only do the cells grow in pavement formation, but they seem to have a tendency to arrange themselves in a structure resembling the acini of glandular tissue (Figs. 2, 4, and 6). No dedifferentiation such as that suggested by Champy⁷ has taken place after 3 months cultivation *in vitro*. The appearance of the cells at the present time is identical with that observed after the first passage. The rate of proliferation is increasing. Every culture doubles in size after 3 or 4 days.

III.

CONCLUSIONS.

1. A strain of pure epithelial cells was obtained from the part of the iris which adheres to the extirpated lens.

2. The optimum condition under which epithelial cells grow *in vitro* is on the free surface of the plasma clot under a film of embryonic tissue juice.

3. The epithelial cells did not dedifferentiate. Although the strain is 3 months old, they grow as a pavement membrane and have kept their epithelial characteristics.

EXPLANATION OF PLATES.

PLATE 11.

FIG. 1. Photograph of a living culture of epithelium 6 weeks old. Shows the characteristic growth for epithelial cells. \times about 135.

FIG. 2. Stained culture of epithelial cells, 6 weeks old. Shows the epithelial pavement formation; mitosis may be seen. A few proliferation centers may be noticed. \times about 660.

PLATE 12.

FIG. 3. Stained culture of epithelial cells, 6 weeks old. To the left may be seen the beginning of an amitotic cell division. \times 1,100.

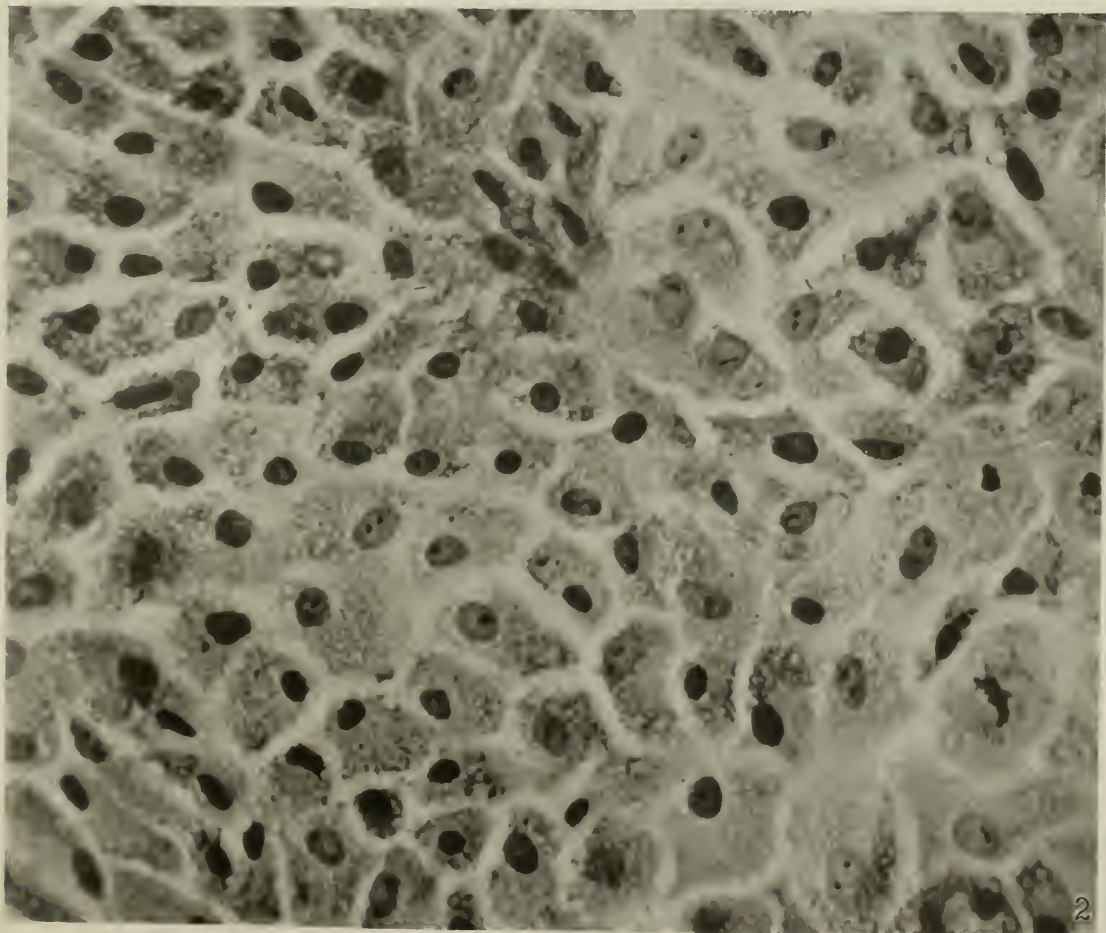
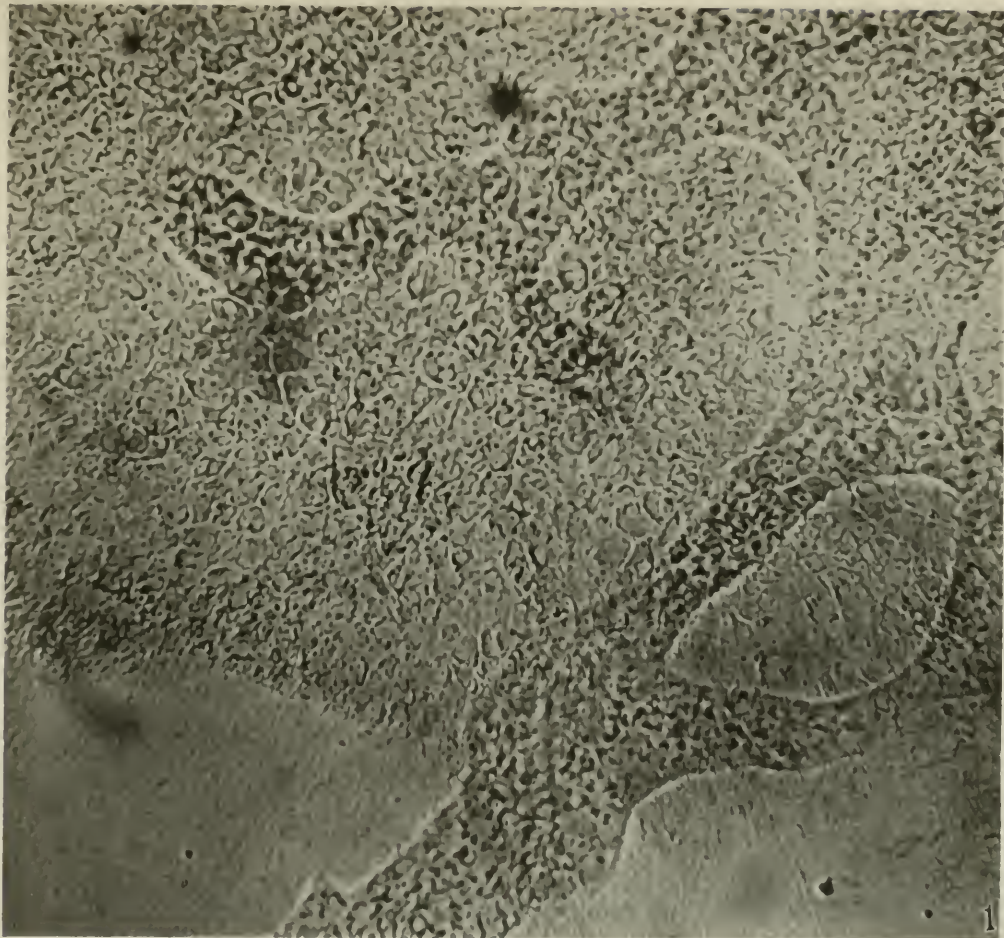
FIG. 4. Stained culture of epithelial cells 2 months old. \times 1,425.

PLATE 13.

FIG. 5. Different stages of mitosis of epithelial cells in cultures.

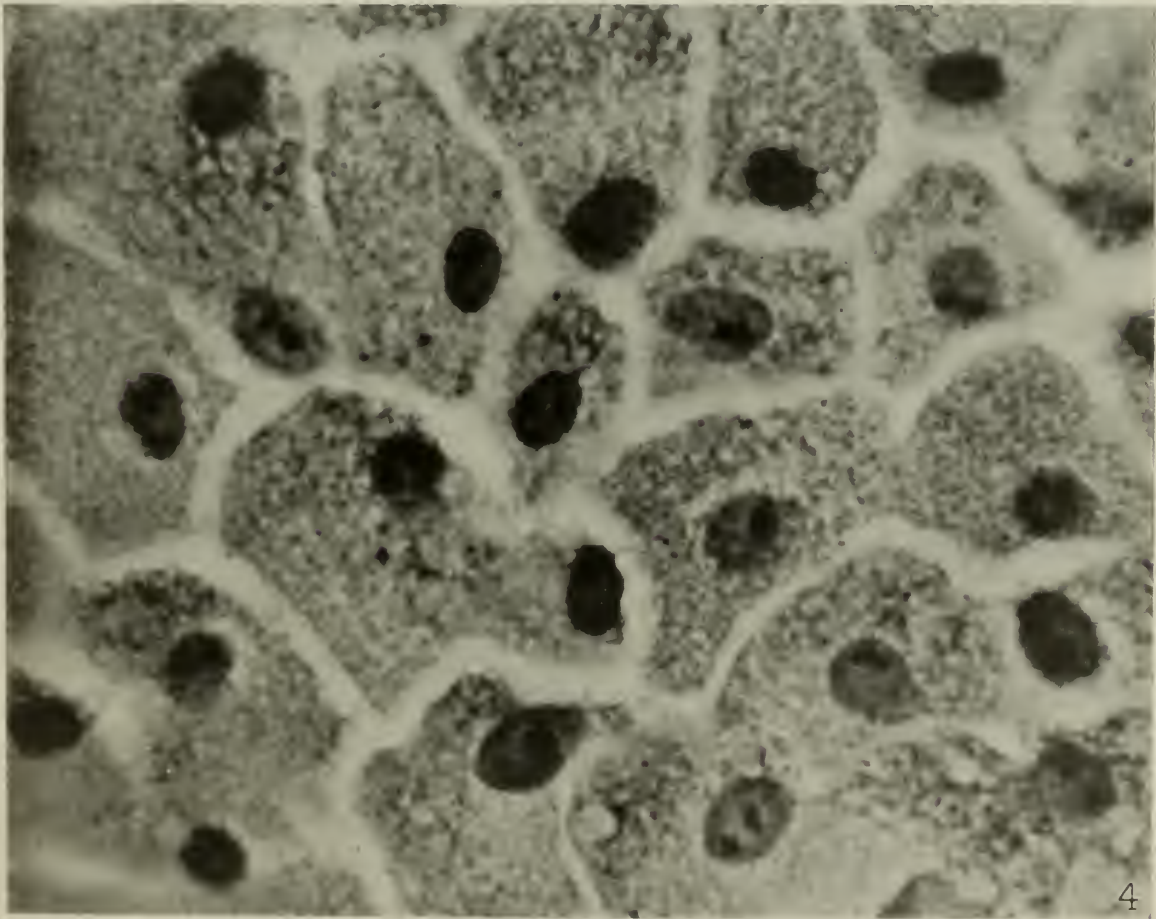
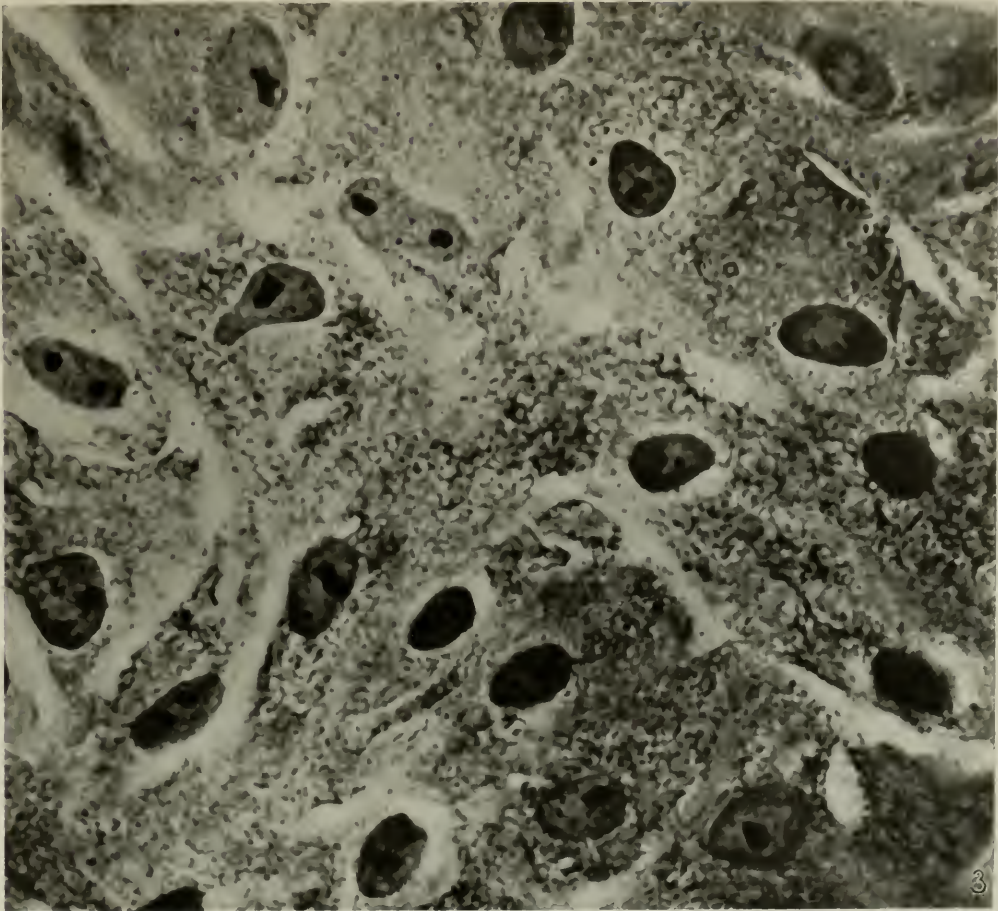
PLATE 14.

FIG. 6. Typical cell arrangement in epithelial cultures. \times 700.

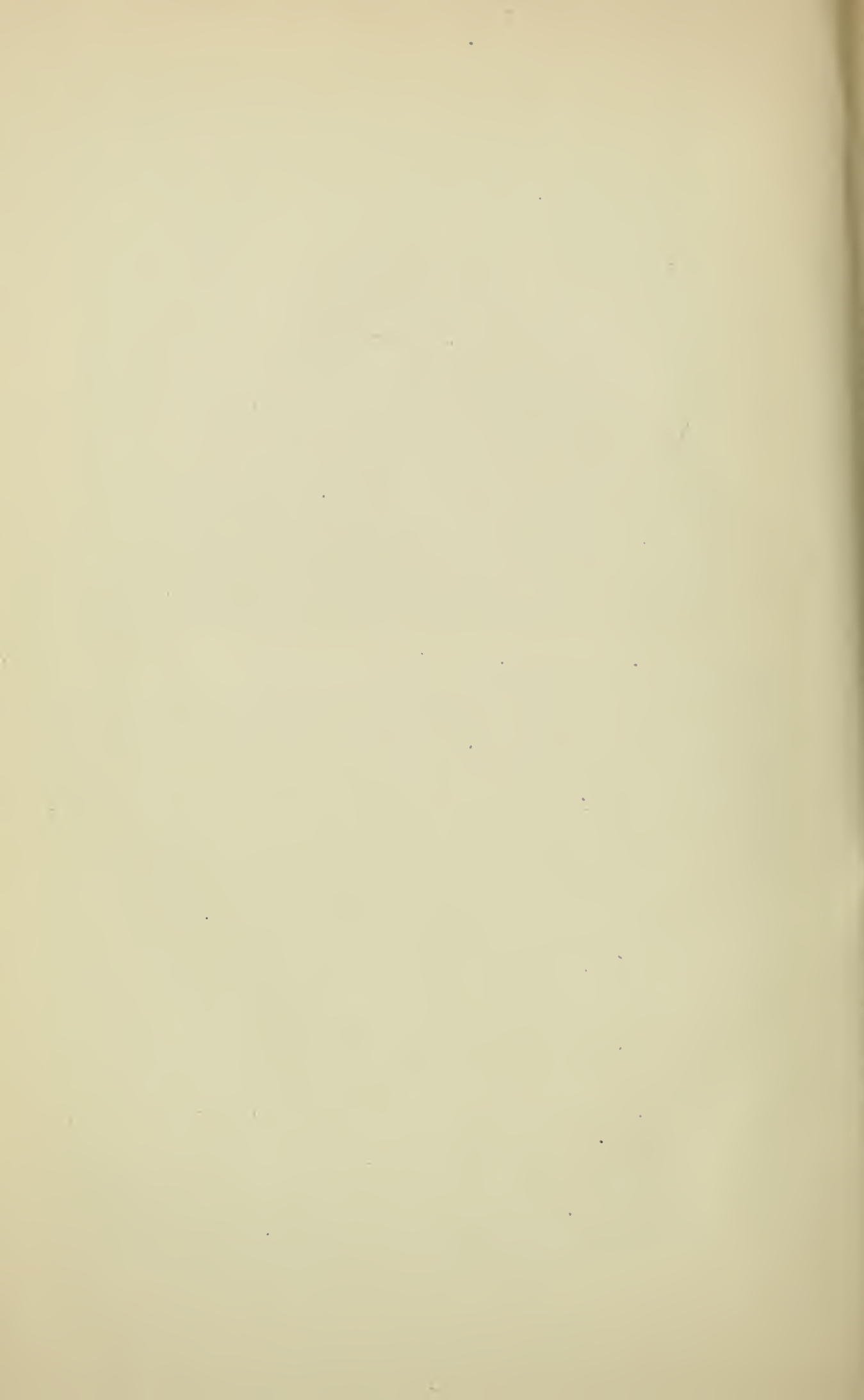


(Fischer: A strain of epithelium.)

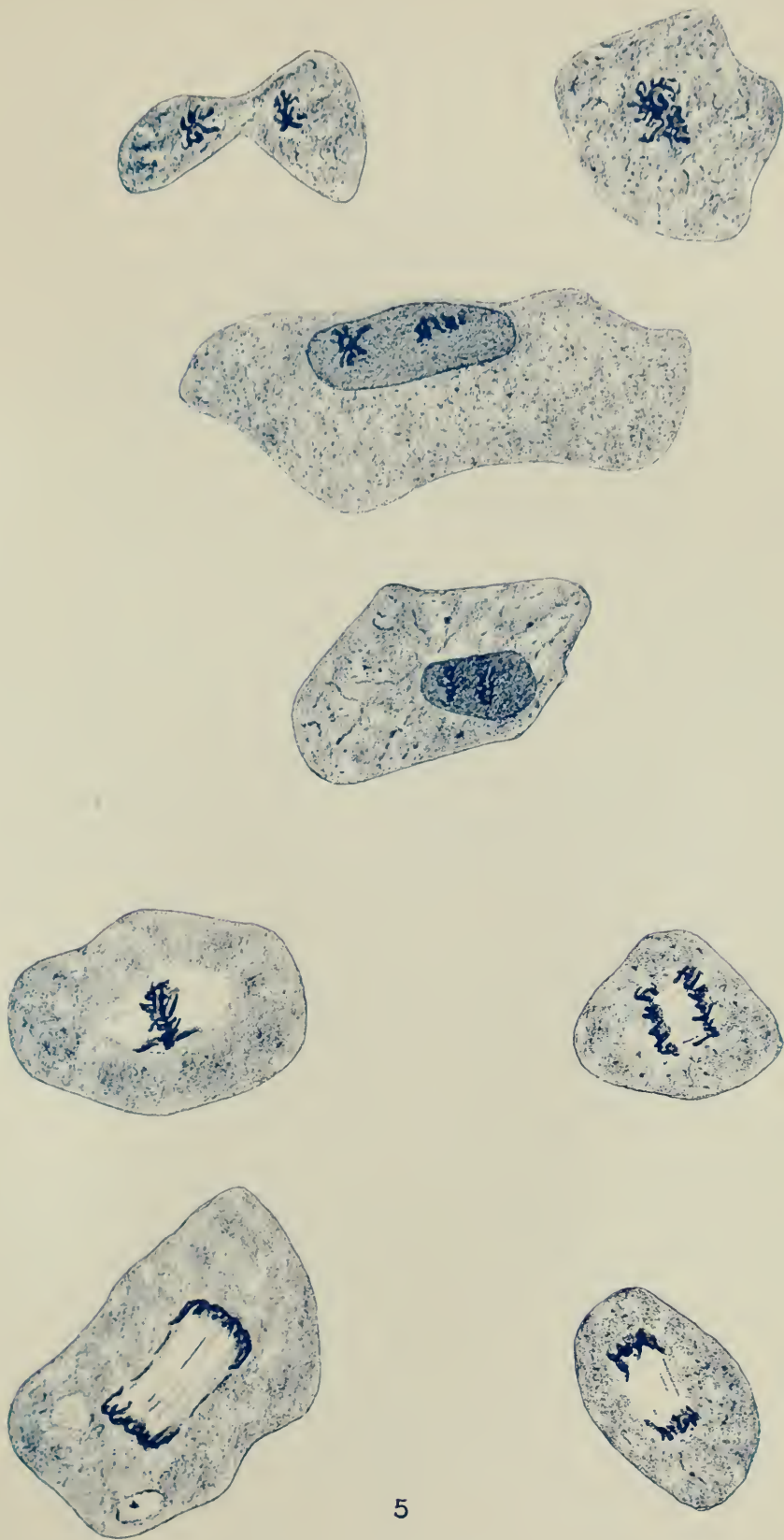
372²

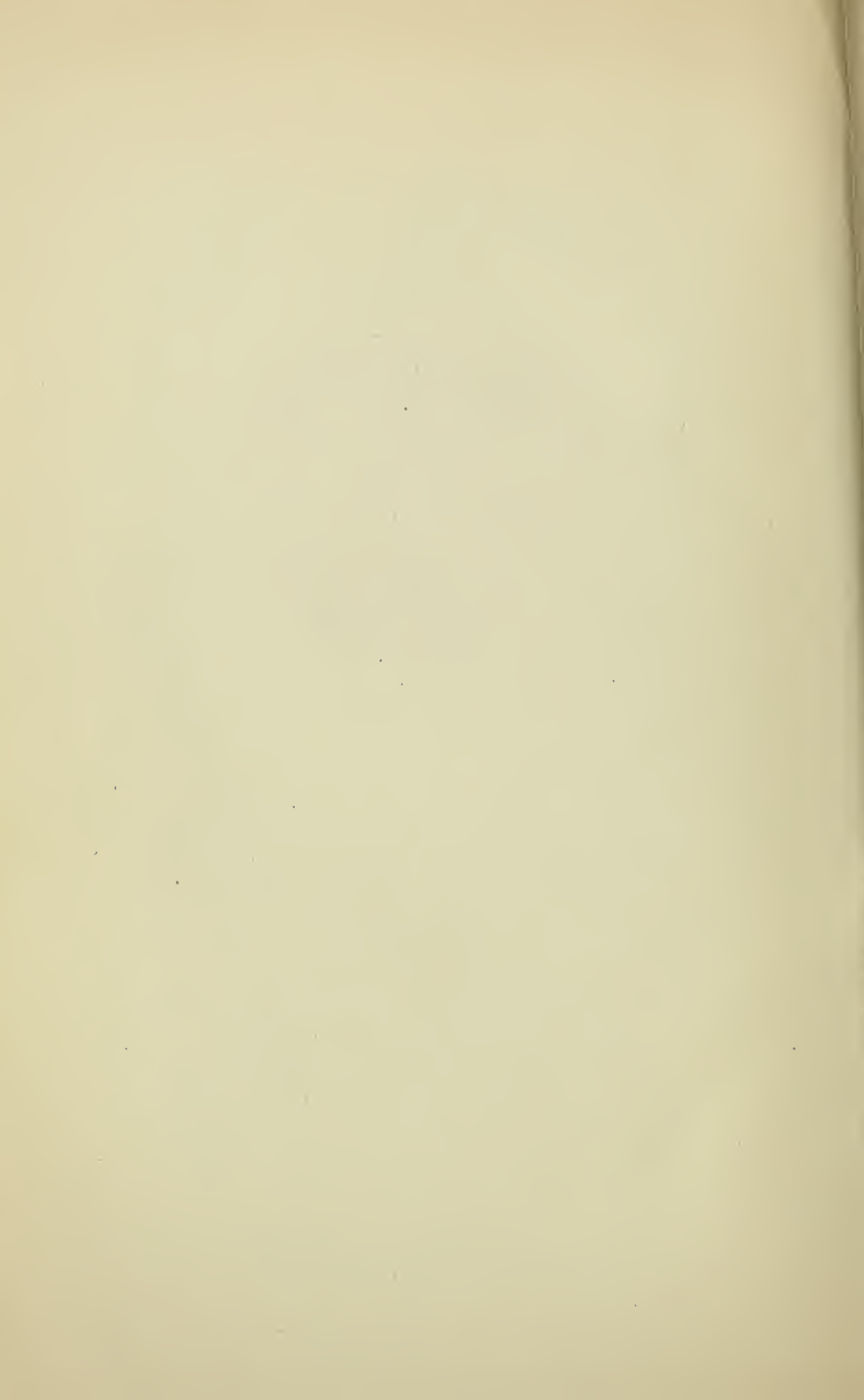


(Fischer: A strain of epithelium.)

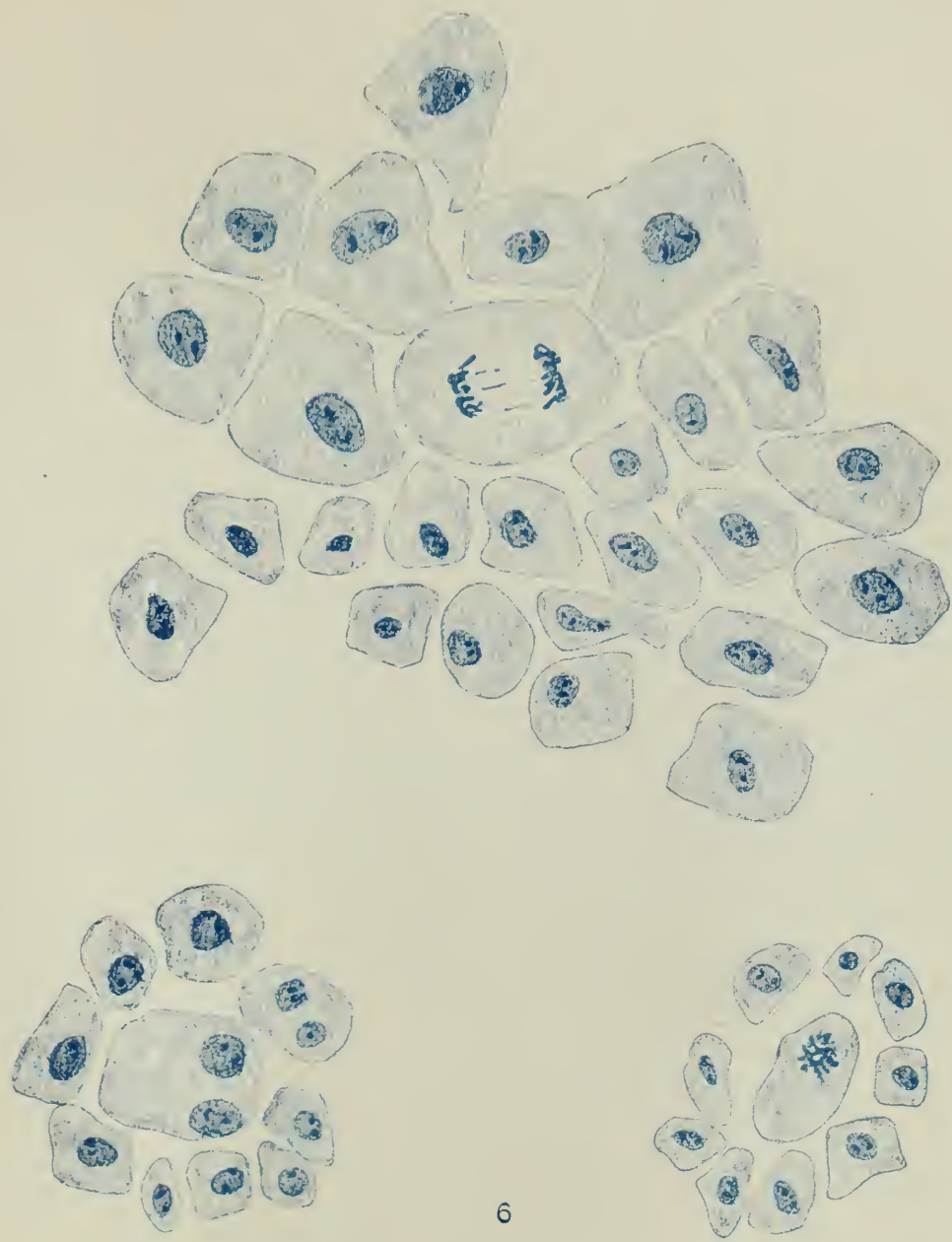


372³



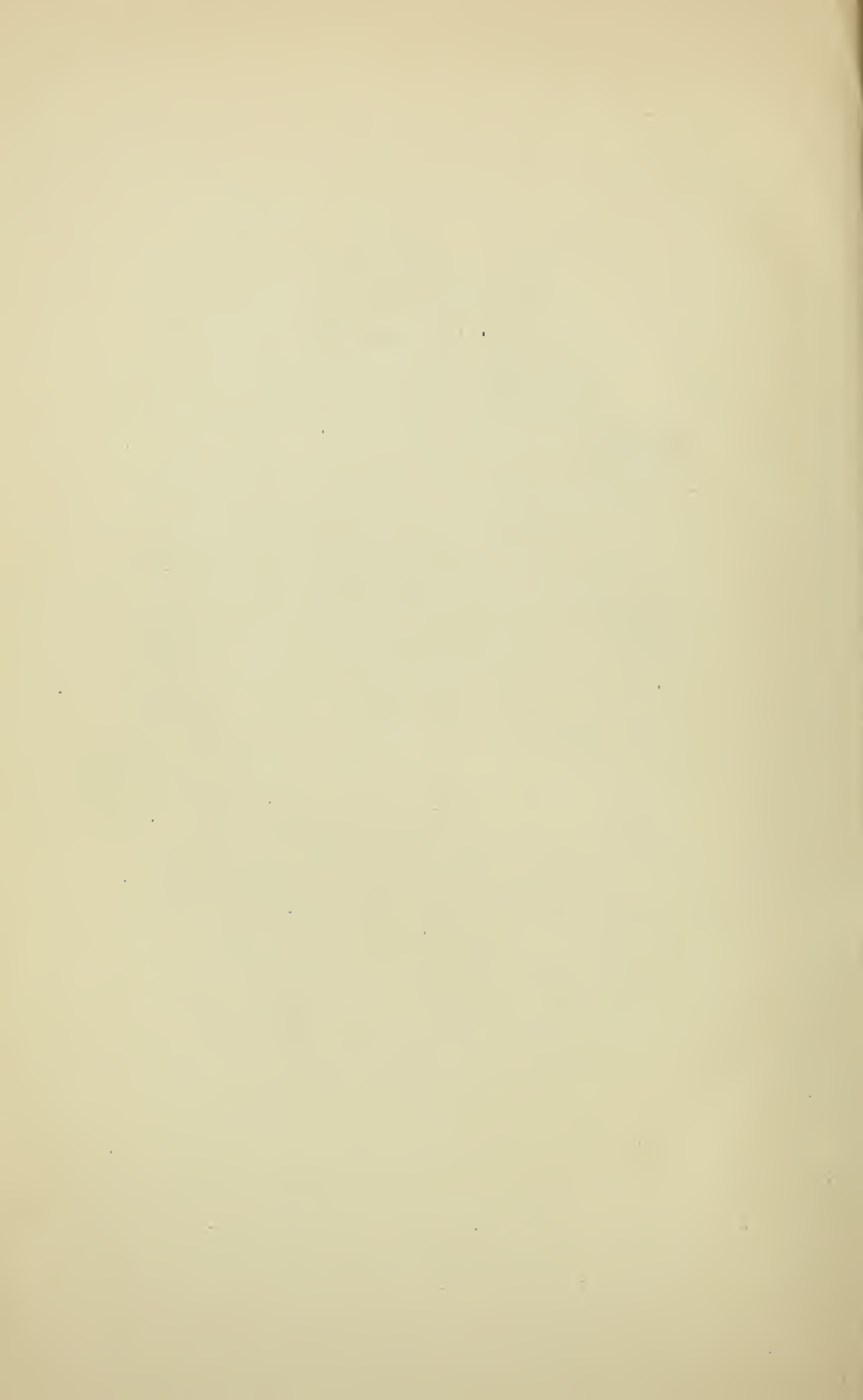


372⁴



6

(Fischer: A strain of epithelium.)



STUDIES ON X-RAY EFFECTS.

IX. THE ACTION OF SERUM FROM X-RAYED ANIMALS ON LYMPHOID CELLS IN VITRO.

BY JAMES B. MURPHY, M.D., J. HENG LIU, M.D., AND ERNEST STURM.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 15.

(Received for publication, October 17, 1921.)

In the course of an investigation on the biological effects of x-rays it was noted that while larger doses of this agent destroy lymphoid tissue, very small exposures, after causing a slight amount of destruction, will bring about an actual stimulation of this tissue.¹ The mechanism of the stimulation phenomenon is of considerable interest owing, among other things, to the relation of the lymphoid tissue to cancer resistance. The most satisfactory stimulation has been obtained with x-rays of comparatively long wave-lengths and, therefore, of low penetrating power. In fact, the best results have followed exposure to the rays from a specially constructed tube with a window which permits the emission of a larger proportion of the soft rays than are given off by the standard tubes; and this tube is operated with a spark-gap of $\frac{1}{2}$ inch.² Of the very small dose used here, approximately 57 per cent is absorbed by the first $\frac{1}{4}$ cm. of tissue, and over 92 per cent before the rays have penetrated to the depth of $\frac{1}{2}$ cm., while at a depth of $1\frac{1}{2}$ cm. only 0.56 per cent of the rays remains. It seems extremely doubtful, therefore, whether these rays penetrate to the deeper lymphoid organs in sufficient strength to bring about any change; yet these organs show as much evidence of stimulation

¹ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

² Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1922, xxxv (in press).

or destruction as do the organs which are superficial enough to be directly acted upon by the rays.

This observation has led to a consideration of the possibility of the spleen and lymph gland changes being secondary to some alteration in the circulating blood or other tissues, brought about by the action of the x-rays. The point is one that has already been the subject of several investigations. Linser and Helber's³ experiments led them to conclude that the serum from x-rayed animals contained a leucotoxin which on injection into other animals produces destruction of the circulating leucocytes. The leucotoxin was destroyed by heating at 55–60°C. The toxin, according to these authors, is transmitted from mother to fetus through the placenta. Capps and Smith⁴ reported similar findings, with the serum from successfully treated leukemia patients, and state that the serum from an x-ray-treated case of leukemia when injected into an untreated case causes a definite fall in the number of white blood cells. Curschmann and Gaupp⁵ also state that serum from x-ray-treated cases of leukemia, in a dilution of 1:100, causes rapid destruction of leucocytes *in vitro*.

Later Klieneberger and Zoeppritz⁶ failed to confirm any of the above experiments, which is true also of other observers⁷ with reference to the so called leucotoxin in the serum of x-ray-treated individuals.

With the evidence at hand indicating the indirect action of the x-rays on the lymphoid tissue, it seemed of interest to reopen the question and to determine whether or not the serum of x-rayed animals has any effect on lymphoid cells *in vitro*.

The Effect of Serum from X-Rayed Animals on Lymphoid Cells in Vitro.

A number of healthy young rats were exposed to a dose of x-rays governed by the following factors: spark-gap 2½ inches; milliamperes 10; distance 12 inches; time 14 minutes. Immediately following

³ Linser, P., and Helber, E., *Deutsch. Arch. klin. Med.*, 1905, lxxxiii, 479.

⁴ Capps, J. A., and Smith, J. F., *J. Exp. Med.*, 1907, ix, 51.

⁵ Curschmann, H., and Gaupp, O., *Münch. med. Woch.*, 1905, lii, 2409.

⁶ Klieneberger, C., and Zoeppritz, H., *Münch. med. Woch.*, 1906, liii, 850.

⁷ Melchner, R., and Wolff, W., *Berl. klin. Woch.*, 1906, xliii, 746.

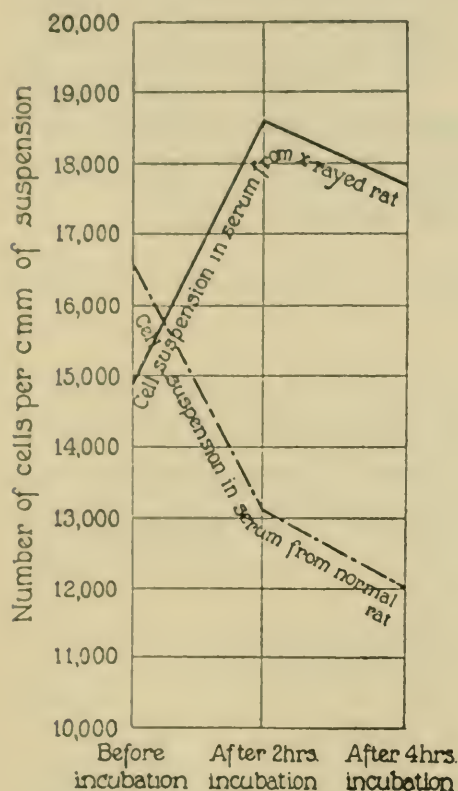
this treatment the animals were anesthetized and exsanguinated by aspiration of the heart. The blood was placed in a test-tube, and after clotting it was centrifuged. The serum was then drawn off and again centrifuged at high speed to remove any remaining cells or fibrin. Serum was collected in the same manner from a like number of normal rats. After the blood had been drawn from the normal rats, the thymus and mesenteric lymph glands were removed under aseptic conditions. The glands were freed from adherent tissue and were then divided as nearly as possible into equal parts so that two lots were made, each having half of the thymus and half of the mass of mesenteric lymph glands from a number of different rats. One of the portions was then mixed with the serum from normal rats and the other with serum from the x-rayed rats and each was then ground thoroughly in a mortar, and the resulting suspensions were passed through filter paper under suction to remove the fibrous tissue and cell clumps. Counts were made of the two filtrates to determine the number of cells present, and then enough of the two sera was added to reduce the counts to between 10,000 and 20,000 cells per c. mm. Another count was made on the suspensions after they had been well shaken to standardize the suspension. The tubes were tightly plugged and placed in a water bath at 37°C. for 2 hours. They were then removed, well shaken, and counted, and again after 4 hours in the water bath this procedure was repeated. Films were made at the time of each count and stained with Wright's blood stain. The enumeration in each case was made by two individuals on different samples of the suspension and when there was a divergence, the mixtures were reshaken and the counting was repeated. Many counts were checked up with the high power lens of the microscope so as to make sure that fragments and debris were not included.

Table I gives the tabulated results of fourteen such experiments in which Serum A is from normal animals and Serum B from x-rayed animals.

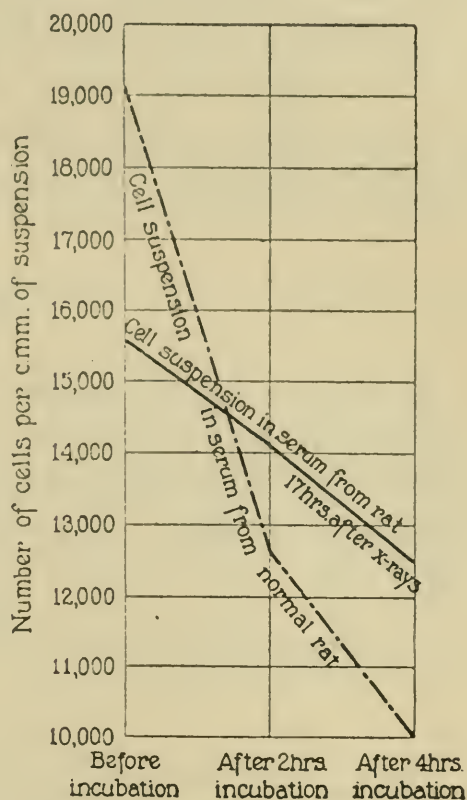
The average of these fourteen experiments (Text-fig. 1) shows that the cells suspended in normal serum decreased by over 3,000 during the first 2 hours and by another thousand by the end of the 4 hour period. The cells in the serum from x-rayed animals increased by over 3,000 cells in the first 2 hours and showed only a slight drop be-

tween the 2 and 4 hour periods. At the end of the period of observation the counts showed the suspensions still had some 3,000 cells more per c. mm. than the original suspension.

Examination of a large number of stained films made from these suspensions at the 2 hour period showed among the cells suspended in serum from x-rayed animals a fairly large number of mitotic figures (Fig. 1). The average was a little less than one mitosis to a thin film,



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Graphic representation of the average of Experiments 1 to 14.

TEXT-FIG. 2. Graphic representation of the average of Experiments 15 to 17.

and occasionally three or more were found in a film. In only one instance was a dividing cell found in the normal serum suspension. The amount of disintegration of the cells, judged by the number of degenerated forms found in the smears, is just as rapid in the serum from x-rayed animals as in that from normal animals. Apparently, therefore, the proliferation of the cells in contact with serum from x-rayed animals is sufficient to replace not only the disintegrated

cells but also actually to increase the total number. A large number of films prepared from the suspensions before incubation failed to show any mitotic figures, thus ruling out the question of the dividing cells being carried over in any appreciable numbers from the glands.

An unsuccessful attempt was made to extend the above observations to rabbits, but the fragility of the lymphoid cells was such that by the end of the 2 hour period no accurate counts could be made. The cells of guinea pigs showed less tendency to disintegrate and a small increase in the number of cells suspended in serum from an x-rayed animal was noted. However, the rate of disintegration was too rapid to obtain definite or consistent results. Finally, rat lymphoid cells suspended in serum from rabbits were destroyed so rapidly as to make it impossible to secure accurate counts.

The Duration of the Stimulative Quality of Serum from X-Rayed Animals.

In order to test the length of endurance of the stimulative effect of the serum from x-rayed rats, the above experiments were repeated, except that in this series the blood was taken 17 hours after the x-ray treatment was given. The results of these observations are given in Table II.

TABLE II.

Time.	No. of cells in suspension.					
	Experiment 15.		Experiment 16.		Experiment 17.	
	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.
Before incubation.....	23,700	15,500	14,880	17,480	19,040	14,100
After 2 hrs. incubation.....	14,080	14,350	11,440	15,240	13,500	12,700
“ 4 “ “	10,080	14,750	8,350	13,250	12,200	12,500

Serum A is from normal rats, Serum B from rats 17 hours after a dose of x-rays.

It will be seen that active stimulative effect of the serum from x-rayed rats is lost by 17 hours after the treatment (Text-fig. 2), but it may be noted that the rate of disintegration is retarded somewhat in the serum from the x-rayed animals. It is not clear whether the retarding action represents an actual slowing down of the disin-

tegration rate or whether there is enough stimulation substance remaining to bring about a less rapid fall in the count by cell multiplication. The finding of one mitotic figure in the comparatively small number of preparations studied suggests the latter possibility.

The Effect of Serum X-Rayed in Vitro on Lymphoid Cells.

A quantity of serum was prepared from normal rats in the same manner as in the preceding experiments. Half of this was exposed directly to x-rays in the same amount as that given to the animals in the previous experiments.

The cell suspensions were prepared in the same manner as described above with the normal and x-rayed serum. The results of these experiments are given in Table III.

TABLE III.

Time.	No. of cells in suspension.					
	Experiment 18.		Experiment 19.		Experiment 20.	
	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.
Before incubation.....	19,100	16,000	17,500	15,200	9,850	13,360
After 2 hrs. incubation.....	13,400	14,100	14,450	14,350	6,300	11,960
" 4 " "	11,000	14,100	13,300	13,300	5,300	10,900

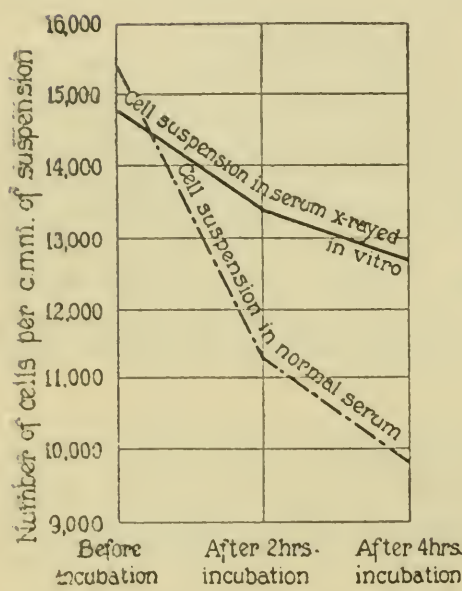
Serum A is from normal rats, Serum B is normal rat serum exposed to x-rays *in vitro*.

Thus the serum x-rayed *in vitro* proved to be devoid of stimulative effect on the suspended lymphocytes, but as in the preceding experiment there was a retardation of fall in the cell count (Text-fig. 3).

Variations in the Response of Lymphoid Cells to Stimulative Effects.

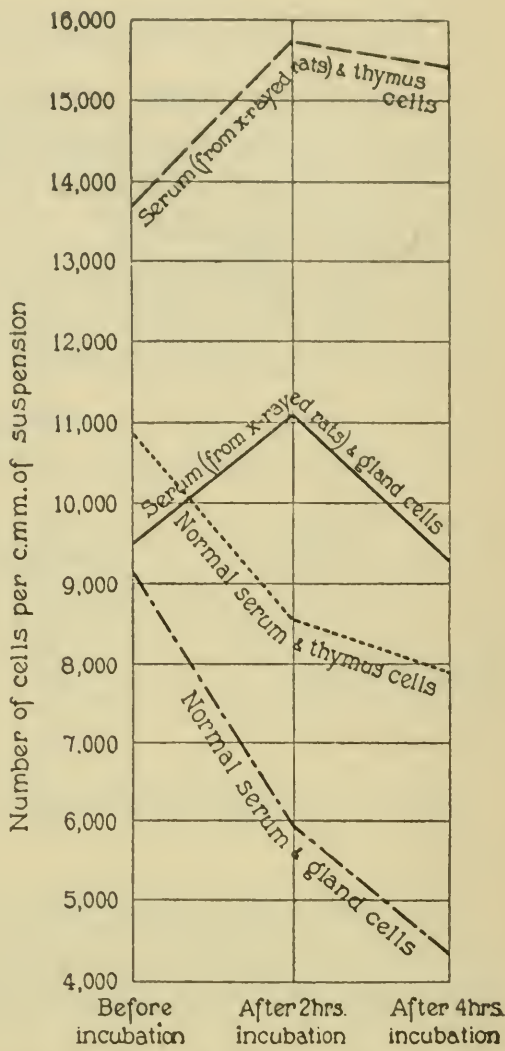
In all the experiments described above, the cell suspensions were prepared from the thymus and mesenteric lymph glands usually of five rats. Each mass of glands was divided into two parts so that the final suspensions contained about equal amounts of the tissue from each animal used in the experiment. It will be noted in the figures given that there was considerable variation in the amount of stimulation in the various experiments, although the dose of x-rays was the

same throughout. Hence a test was made to ascertain whether the cells of the thymus and the lymph gland shared equally in the stimulation and also whether there was striking individual variation in the degree of response between cells of different animals.



TEXT-FIG. 3.

TEXT-FIG. 3. Graphic representation of the average of Experiments 18 to 20.



TEXT-FIG. 4.

TEXT-FIG. 4. Graphic representation of the average of Experiments 21 to 25.

Serum from normal and x-rayed rats was prepared according to the method described in the first series of experiments. The thymus from two rats and the lymph glands from the same two animals were prepared separately so as to yield a suspension of thymus cells in serum from x-rayed rats, and a suspension of thymus cells from the same

TABLE IV.

Time.	No. of cells in suspension.											
	Experiment 21.				Experiment 22.				Experiment 23.			
	Serum A + lymph glands.	Serum A + thymus.	Serum B + lymph glands.	Serum B + thymus.	Serum A + lymph glands.	Serum A + thymus.	Serum B + lymph glands.	Serum B + thymus.	Serum A + lymph glands.	Serum A + thymus.	Serum B + lymph glands.	Serum B + thymus.
Before incubation....	11,450	4,000	9,900	9,500	9,000	11,400	18,450	14,350	8,450	14,250	5,200	14,250
After 2 hrs. incuba- tion.....	7,600	2,550	10,450	12,030	6,350	9,160	22,500	17,700	5,970	10,150	6,970	12,800
After 4 hrs. incuba- tion.....	6,500	2,150	4,850	13,350	3,800	8,400	22,000	17,800	3,850	9,650	6,500	12,300
									7,850	14,100	4,400	16,500
									4,000	12,400	4,300	20,600
									3,300	11,500	3,700	18,150

Serum A is from normal rats, Serum B from rats immediately after a dose of x-rays.

animals in normal serum. Two like suspensions of the lymph glands from the same animals in the two sera were prepared for comparison. Table IV shows the result of four such experiments.

Another like experiment was carried out, to test the response of glands from different groups of rats to the same serum from x-rayed animals.

The figures for these experiments are given in Table V.

TABLE V.

Time.	No. of cells in suspension.					
	Experiment 25.					
	Serum A + lymph glands.	Serum A + thymus.	Serum B + thymus.	Serum B + lymph glands (1).	Serum B + lymph glands (2).	Serum B + lymph glands (3).
Before incubation.....	16,970	14,000	10,200	17,800	2,800	3,750
After 2 hrs. incubation.....	11,600	9,100	11,840	18,800	4,360	4,150
“ 4 “ “	11,070	8,450	12,500	16,000	3,950	4,200

Serum A is from normal rats, Serum B from rats immediately after a dose of x-rays.

It is obvious from these experiments that the thymus and lymph gland cells are affected about equally by the serum from x-rayed animals (Text-fig. 4), although the thymus cells from some animals respond more readily than the lymph gland cells from the same animals, while in others the opposite is true.

There is also considerable variability in the stimulative power of the same serum on the lymphoid cells of different individuals.

The Effect of the Serum from Animals after a Very Large Dose of X-Rays on Lymphoid Cells.

In further experiments, an attempt has been made to determine whether there is a destructive action on the lymphoid cells of serum from animals after a very large dose of x-rays. Rats were exposed for an hour to a dose of x-rays, otherwise governed by the same factors as in the preceding experiments, and the effect of the serum of these animals was tested on lymphoid cell suspension. There was no

evidence of a stimulative effect, nor was there any more rapid disintegration of the cells than was observed to take place in the normal serum.

DISCUSSION.

The experiments reported here fail to show any evidence of the presence of a so called lymphotoxin in the serum of x-rayed animals, even after an exposure so large as to cause almost complete destruction of the lymphoid tissue of the living animals. It is true, however, that these experiments are not an exact repetition of the earlier work along this line, but it seems probable that if any such leucotoxic substance was present in the serum of x-rayed animals, some indication would have appeared among our results. It is difficult to conceive of a lymphotoxin of such power as to be effective in dilutions of 1:100 resulting from a comparatively small dose of x-rays given to a leucemic patient. It is much more difficult to judge the reported results of the injection of serum from an x-rayed individual into animals, for there is the complicating effect of the foreign protein reaction to be taken into account, as well as the instability of the blood counts of the rabbit and guinea pig, the animals used for these tests. In regard to the latter point our experience has been that it is necessary to resort to extreme measures of precaution in order to get a fairly stable blood picture in such animals.

The source and character of the stimulus for lymphoid cells contained in the serum from x-rayed animals are questions about which there is as yet little to be said. The fact that this stimulative quality is not possessed by serum x-rayed *in vitro* suggests that the change is not a simple one in the serum itself. Furthermore, it is known that the stimulation of lymphocytes induced by x-rays *in vivo* is always preceded by a certain amount of destruction of lymphoid cells, a fact suggesting the possibility of the stimulating substance being of the nature of a disintegration product of lymphoid cells.

There is ample proof, both from the cell counts and the presence of mitotic figures, that multiplication actually takes place in cells in a fluid medium, although it has generally been supposed that a matrix of some kind is a necessity for growth. No other explanation of the results described is apparent than that cells are capable of being

stimulated to active multiplication in a fluid medium, and that such a stimulative agent is present in the serum of x-rayed animals. How great a part this agent plays in the stimulation observed to take place *in vivo* after a small dose of x-rays is still to be determined.

SUMMARY.

Lymphoid cells, prepared from the thymus and lymph glands of rats, when suspended in the serum of x-rayed rats and incubated for 2 hours, increase in number from 15 to 30 per cent, and mitotic figures are found among these cells in fairly large numbers. A like suspension of cells in normal serum undergoes rapid disintegration and in only one instance among a large number of films examined was a mitotic figure found.

The stimulative effect of the serum from x-rayed rats endures from 1 to 2 hours after the exposure but is not detectable in the serum taken 17 hours or later after the treatment. Serum x-rayed *in vitro* is devoid of stimulative action.

The lymphoid cells of rabbits and guinea pigs are so fragile as to make impossible the obtaining of counts accurate enough for experimental purposes. The serum of one species caused such rapid disintegration of the cells of another that it was impossible to determine the specificity of the reaction.

EXPLANATION OF PLATE 15.

FIG. 1. Mitotic figures found among the cells suspended in serum from x-rayed animals.

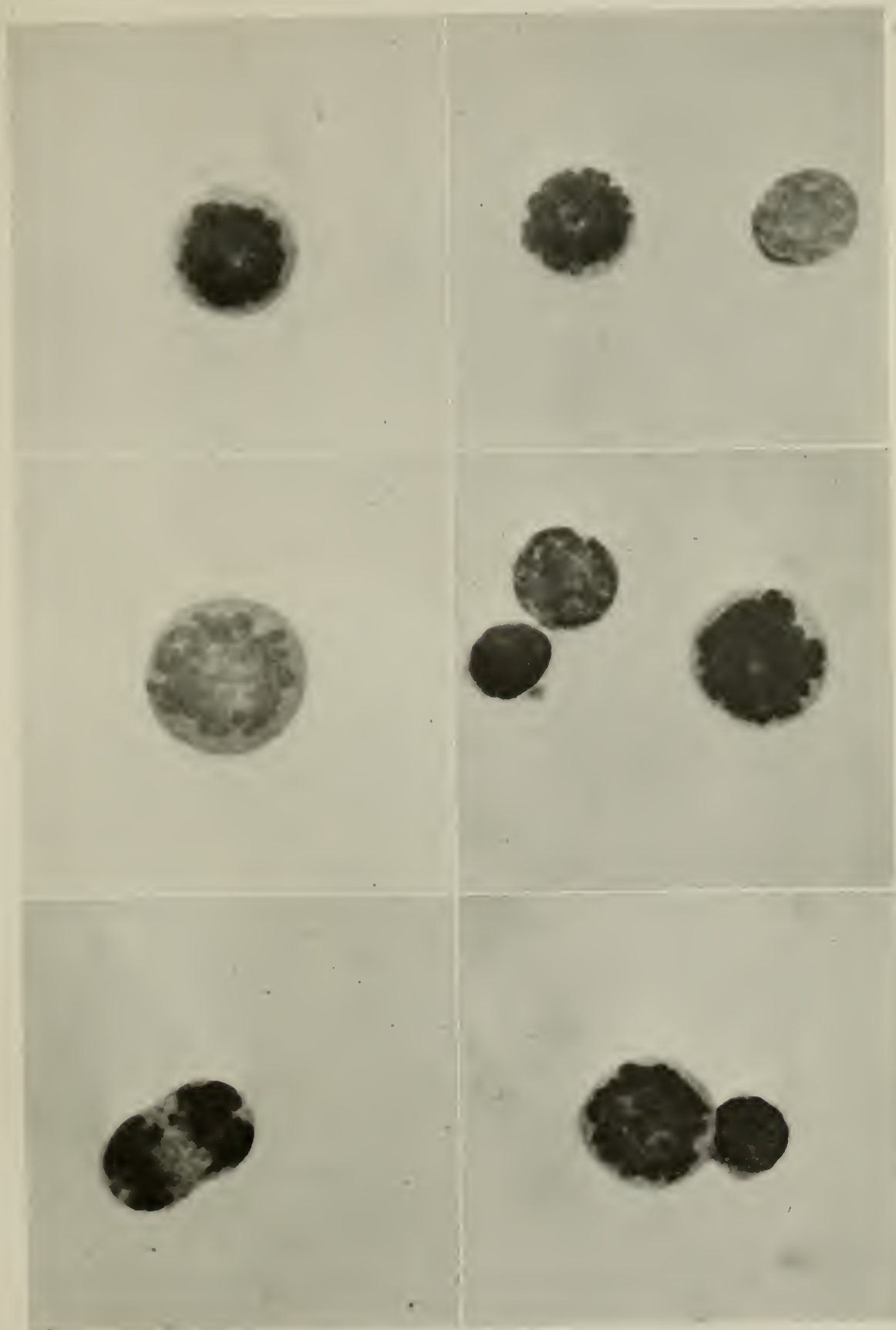
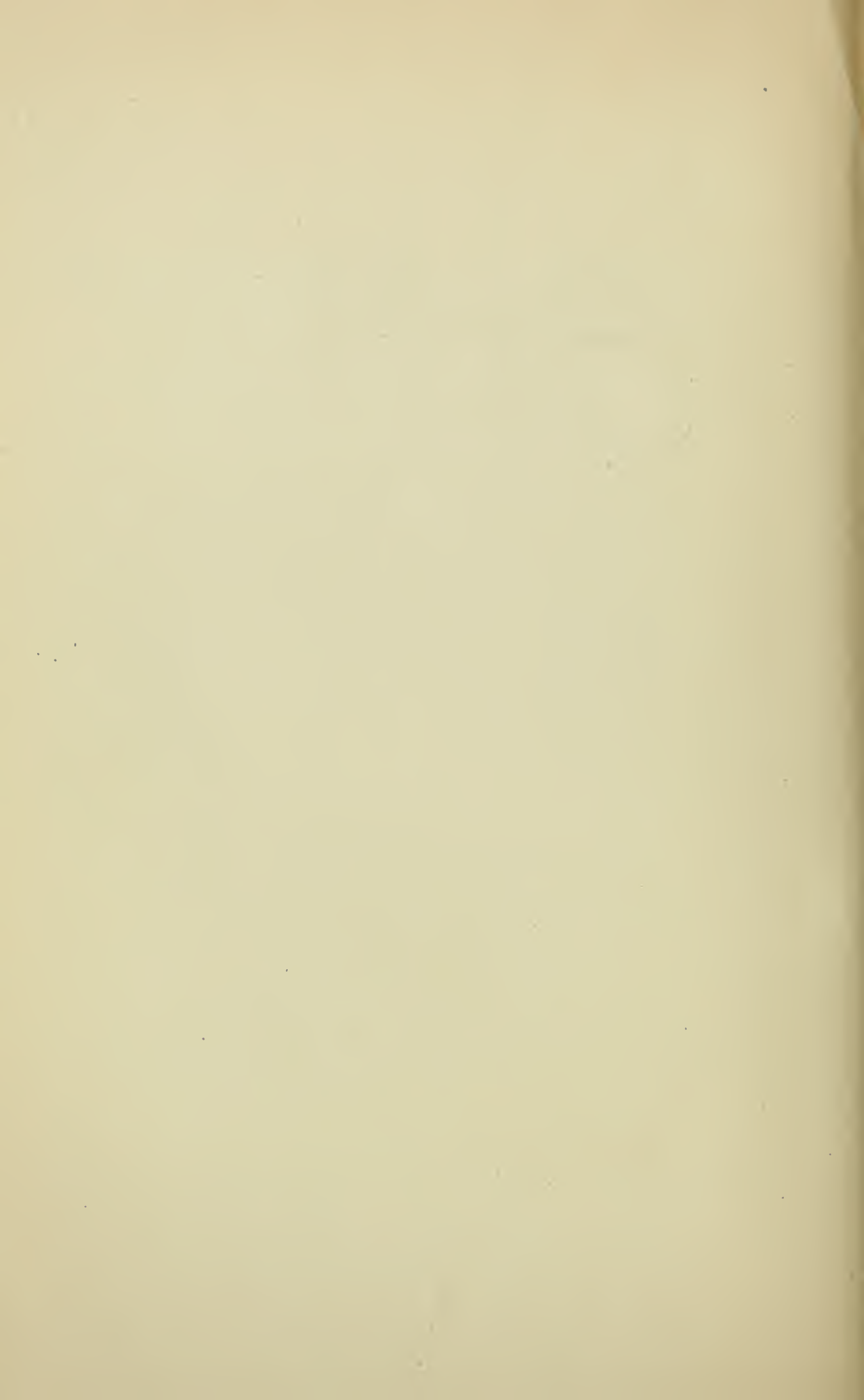


FIG. 1.

(Murphy, Liu, and Sturm: Studies on x-ray effects. IX.)



THE MEASUREMENT OF INTRAVENOUS TEMPERATURES.

By HARRY CLARK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 16.

(Received for publication, October 20, 1921.)

The temperatures of various parts of the body, as determined with the ordinary clinical thermometer are, in general, different, and the differences are not always consistent. We cannot assume, therefore, that such measurements give a true index of the mean internal temperature of the body, which may perhaps best be identified with the temperature of the blood flowing in large blood vessels. Moreover the instrument itself is not free from objection. Its heat capacity is sufficiently great to lower appreciably for some time the temperature of tissue with which it comes in contact, and its response is too slow to follow fluctuations found with more sensitive apparatus. Although measurements made with a clinical thermometer are doubtless quite satisfactory for most purposes, it is possible that, for certain kinds of experimental work, fairly accurate determinations of blood temperatures may prove interesting. Apparatus which has been devised for this purpose by the author is described below.

The temperatures are measured by a thermoelectric method, in which one makes use of the fact that the discontinuity of electric potential at a junction of two dissimilar metals depends only on the nature of the metals and the temperature of the junction. A closed circuit of two metals contains, of course, two opposed junctions. If the second of these junctions is kept at a known constant temperature, the resultant thermoelectric force in the circuit is the difference between the potential discontinuities at the two junctions, and this is a function of the temperature of the first junction only.

This thermoelectromotive force may be used in either of two ways to measure temperature. It may be measured directly by means of

galvanometer, potentiometer, and standard cell, or it may be allowed to maintain a current in the circuit which, according to Ohm's law, will be proportional to the voltage, and which may be measured by the galvanometer deflection. Each of these ways has certain advantages. In the present case the temperature range is small, being only about 5°C. above and below normal, which may be taken as 37.5°C., and the resistance of the circuit is fixed. For these reasons, and in the interest of simplicity and quickness of operation, the galvanometer deflection method was adopted. To use this method to best advantage the second thermocouple is kept constantly at 37°C. by means of a special thermostat.

The apparatus consists essentially of the unit containing the couple to be placed in the body, which is referred to as the needle unit; the portable thermostat; the cable connecting the needle unit with the thermostat; and the galvanometer with necessary switches, etc. Each of these parts is described below in detail.

The Needle Unit.

The longer unit shown in Fig. 1 is the needle unit. The couple consists of two No. 40 single-silk-covered wires, one of copper, the other of constantan, contained in the central tube of spring steel which has an external diameter of 0.7 mm. and an internal diameter of about 0.3 mm. At the needle end the wires are connected together and to the tube with solder. The technique of making this soldered joint required considerable study. It was found that a good seal could be made without allowing the solder to join the two wires together or to the tube over a length of more than 1 mm. At the other end the wires are soldered to the copper screw and the constantan center pin of the connecting plug. The steel tube lies within a hypodermic needle (external diameter 1.4 mm.), sufficient space being left between them to allow blood to flow into the glass pipette. A flow of blood shows that the needle has been properly inserted into the blood vessel and brings the needle quickly to temperature equilibrium. In the figure the tube containing the couple is shown protruding from the needle, but in practice it will be withdrawn to the best position by means of the adjusting screw. All metal parts except the electrical contacts are nickel-plated and polished. The two

units shown in Fig. 1 are interchangeable, the resistance of each being 21.86 ± 0.02 ohms and the correction factor for lack of uniformity of the constantan being about 1.002.

The Flexible Cable.

Either of these units is connected to the couple contained in the thermostat by means of a flexible cable about 4 meters long. The copper and constantan conductors consist each of several No. 40 wires, each group sewed up in narrow silk ribbon. They are surrounded by a helical spring 4 mm. in diameter of piano steel wire which is covered with diagonally woven silk braid. This cable is not injured by crumpling or pulling, and is so light and flexible as not to interfere with the operator's use of the needle. The resistance is 34 ohms. The ends are provided with small receptacles, the terminals of which are of copper and constantan, to fit the connecting plugs of the needle unit and the thermostat. These receptacles also serve to connect the external metal parts of the needle unit through the steel spring of the cable to the ground plate of the thermostat.

The Thermostat.

The thermostat is shown in Figs. 2 and 3. *A* and *B* are the walls of a flask of Pyrex glass, the space between which is filled with mercury which serves both as the constant temperature bath and, by its change of volume and consequent change of level in tube *D*, as the temperature regulator. The flask is wrapped in several layers of asbestos paper through the second one of which is sewed the resistance wire for warming the bath electrically. One end of this wire is connected to a binding post on top of the thermostat and to the metal sleeve *E* which is cemented to the glass tube *D* and which carries the adjusting screw *F* with its sharp pointed platinum wire through which contact with the mercury is made. The other end of the resistance wire is connected to another binding post and to the platinum wire sealed into the flask at *L*. The flask with its wrapping sets in an ordinary pint thermos bottle, the space above the flask being filled with non-conducting material. The thermocouple consisting of the constantan wire from plug *H* and the copper wire from a copper

binding post is set in wax in the glass tube *C*. The space between *B* and *C* contains mercury, above which is a packing of cloth. The copper screw of plug *H* is connected to another copper post on top of the thermostat. All posts and other metal parts having to do with the thermoelectric circuit are attached to the upper disc, *I*, of hard rubber, while the posts for heating current are set in the lower disc *K*. The metal plate *J* between these discs may be grounded. The heating current, therefore, cannot leak into the thermoelectric circuit. The cap *G* protects the screw *F* from accidental change of adjustment.

The resistance wire for the heating current has a resistance of about 28 ohms and is operated from the 110 volt lighting circuit through an external series resistance consisting of a 25 watt, 110 volt lamp. When the mercury makes contact with screw *F* the heating current is short-circuited through the mercury. With the regulator set for a temperature of 37°C., the room temperature being 20°C., the heating is *on* about 6 seconds and *off* about 10 seconds. A calculation shows that the mean fluctuation in temperature of the mercury bath could not exceed 0.02°C. under the worst conditions, but since the heat is introduced at the outer surface of the flask—the only place where heat can escape from the flask—there is no consistent flow of heat through the mercury and therefore probably only a negligible fluctuation of temperature at the middle, and this is further decreased by the presence of the mercury between *B* and *C*. The use of the mercury contact to short-circuit the heating current rather than to operate a relay has a distinct advantage in that there is no arc when the contact is broken. The currents and voltages which can be handled in this way without arc are, however, small and had to be found by preliminary experiments. This thermostat has been run almost continuously for several months and though the mercury surface is exposed to air, it is still perfectly clean and bright. Its action has been studied with a high sensitivity galvanometer and no temperature fluctuations have been found.

The Galvanometer.

A Leeds and Northrup type R galvanometer 2500-a is used, since the sensitivity is such that the desired range of temperature covers the 50 cm. scale at a distance of 1 meter, the damping is critical, and the

period is only 5 seconds. It is mounted in a box backed by a grounded metal plate which is fastened to the wall. The galvanometer is packed in cotton and the box provided with copper posts for external connection.

The external circuit from the thermostat through the switches to the galvanometer, including binding posts, wire terminals, etc., is of copper. A 50 ohm copper resistance is mounted with the switches to provide critical damping for zero readings, and to correct for slight stray thermoelectric forces in the galvanometer. The switch is mounted on a metal ground-plate. The insulated connecting wires are protected against leakage by being wrapped with copper wires fitted with terminals for connection to the ground-plates of switch, galvanometer, and thermostat.

Preliminary Tests of the Apparatus.

Calibration curves were taken with a large water bath, stirred by a motor, the temperatures being read with a microscope on a Bureau of Standards thermometer divided to 0.1°C . The curves are smooth and consistent to within 0.01°C . save for a few isolated points the worst of which are within 0.02°C . of the curve. The apparatus is therefore not more in error than the thermometer readings, probably much less. Readings accurate to 0.01°C . are obtained when the needle is immersed in water to a depth of only 3 mm., and equilibrium is reached within the normal time of damped swing of the galvanometer. Placed in the left ventricle of the heart of a rabbit, equilibrium was reached with the same degree of promptness after the flow of blood into the pipette was established. It is therefore safe to assume that the error in intravenous measurements will not be greater than 0.01°C .

EXPLANATION OF PLATE 16.

FIG. 1. The longer unit is the needle unit, the end of which is inserted into the blood vessel. The small tube inside the pipette and needle contains the two wires of the thermocouple. The shorter unit is used for taking mouth temperatures.

FIGS. 2 and 3. The thermostat which maintains the second thermocouple at a constant temperature.

390^a-

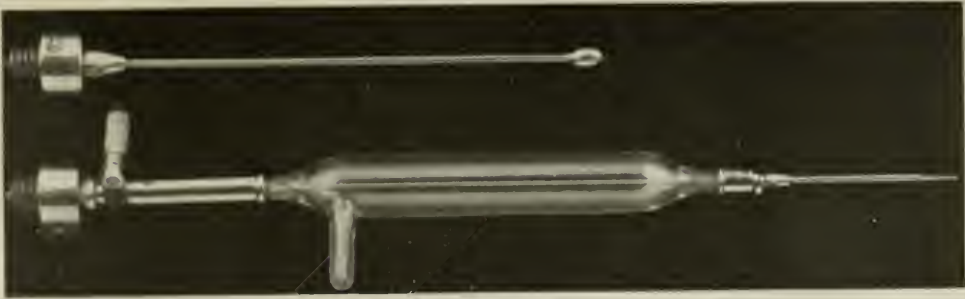


FIG. 1.

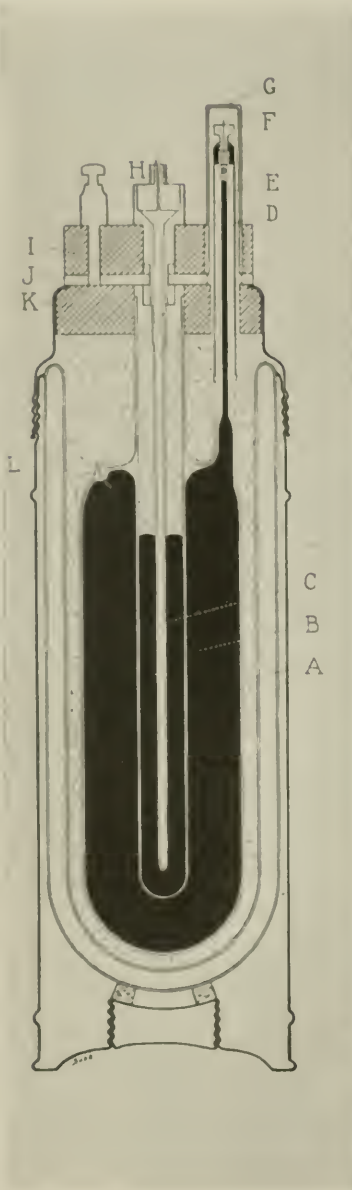


FIG. 2.



FIG. 3.

(Clark: Measurement of intravenous temperatures.)

VENEREAL SPIROCHETOSIS IN AMERICAN RABBITS.

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PLATES 17 TO 20.

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Ross,¹ in 1912, in a discussion of an intracellular phase of *Treponema pallidum*, mentioned a disease of rabbits characterized by chancres, buboes, ulcers on the genitals, mouth, anus, etc., in which he reported finding cell inclusions in the mononuclear leucocytes similar to those which he had found in human syphilis. He suspected that there might be a spiral phase of the parasite of the rabbit disease, and Bayon² undertook the examination of the genital lesions, in which he found a spiral organism indistinguishable from *Treponema pallidum*. Similar lesions appeared in other rabbits 25 days after inoculation of the material containing the spiral organism. Bayon described the rabbit spirochete as being about one and a half times the diameter of a blood corpuscle in length, having 5 to 8 closely set, deep, rather elastic curves, and showing rotating but not lashing movements. He remarked that students of experimental syphilis in the rabbit should take note of the presence of this new species of *Treponema*.

Arzt and Kerl,³ in 1914, reported finding in a peculiar scaly, papular, or erosive lesion, with crust, around the anogenital region of apparently normal rabbits, spiral organisms morphologically indistinguishable from *Treponema pallidum*. Of 850 rabbits examined by them, 26.9 per cent showed the same organism, and in one series of 189 adult rabbits the spirochetes occurred in 34.4 per cent. Arzt and Kerl transmitted the spirochetal affection by applying to the scarified vulva or prepuce of normal rabbits the exudate from the lesion of a rabbit showing the spontaneous disease. After several weeks incubation, the inguinal lymph nodes became somewhat enlarged, and the spirochetes appeared. Similar lesions of lips and clitoris showed the spirochete in large numbers. Intratesticular inoculations were unsuccessful, possibly because of the contaminated nature of the material, and inoculations into the scarified eyebrows of two monkeys were negative.

¹ Ross, E. H., An intracellular parasite developing into spirochaetes, *Brit. Med. J.*, 1912, ii, 1653.

² Bayon, H., A new species of *Treponema* found in the genital sores of rabbits, *Brit. Med. J.*, 1913, ii, 1159.

³ Arzt, L., and Kerl, W., Weitere Mitteilungen über Spirochätenbefunde bei Kaninchen, *Wien. klin. Woch.*, 1914, xxvii, 1053.

With a view to differentiating between this organism and *Treponema pallidum*, Arzt and Kerl^{3,4} inoculated the testicle of a rabbit suffering from the disease in question with a strain of *Treponema pallidum*. The inoculation was successful, but since a syphilitic rabbit can be superinfected or doubly infected, as shown by Truffi,⁵ and by Uhlenhuth and Mulzer,⁶ no conclusion was drawn. No importance was attached to the Wassermann reaction, and an attempt to employ as antigen in a complement deviation test tissue from syphilitic orchitis of the rabbit failed. Examination of certain stocks in Innsbruck,⁷ where no experimental work on syphilis in the rabbit had ever been done, showed the same disease to prevail.

Similar observations were made on Hamburg rabbits by Jacobsthal in 1920.⁸ The lesions present were situated on the vulva and were of a squamous, erosive kind. The dark-field microscope revealed, in the lesions and in the vaginal secretion, numerous spirochetes resembling *pallidum*. Jacobsthal thought the spiral organism to be a trace thicker than *pallidum* and not to exhibit the undulatory movements so characteristic of the latter. Transmission experiments with mice and guinea pigs were negative, but lesions were reproduced in female rabbits in three instances by scarification of the vulva, after an incubation period of 3 to 6 weeks. A chancre-like lesion was noted in one animal. He proposed calling the disease *paralues cuniculi* and the organism *Spirochæta paraluis cuniculi*.⁹

Later in 1920, Schereschewsky¹⁰ stated that the peculiar lesion of the rabbit disease is of papular and ulcerative form and is transmitted by coitus, the incubation period varying from 14 to 30 days.

In 1921 Klarenbeek¹¹ observed spirochetal lesions in five Dutch rabbits. Inoculations of the spirochete-containing material by scarification into the perineal

⁴ Arzt, L., Spirochätenbefunde in Genitalveränderungen ungeimpfter Kaninchen, *Dermat. Z.*, 1920, xxix, 65.

⁵ Truffi, M., Ueber die Empfänglichkeit des Kaninchens gegenüber syphilitischen Reinfektionen, *Centr. Bakt., 1te Abt., Orig.*, 1910, liv, 337.

⁶ Uhlenhuth, P., and Mulzer, P., Beiträge zur experimentellen Pathologie und Therapie der Syphilis mit besonderer Berücksichtigung der Impf-Syphilis der Kaninchen, *Arb. k. Gsndtsamte*, 1913, xlv, 307.

⁷ Arzt, L., and Kerl, W., Zur Frage der "Kaninchensyphilis," *Dermat. Woch.*, 1920, lxxi, 1047.

⁸ Jacobsthal, E., Untersuchungen über eine syphilisähnliche Spontanerkrankung des Kaninchens (*Paralues cuniculi*), *Dermat. Woch.*, 1920, lxxi, 569.

⁹ Arzt and Kerl⁷ remind Jacobsthal that neither incubation time nor histological changes of the lesion warrant the differentiation of this disease from experimental syphilis in rabbits.

¹⁰ Schereschewsky, J., Geschlechtlich übertragbare originäre Kaninchensyphilis und Chinin-Spirochätotropie, *Berl. klin. Woch.*, 1920, lvii, 1142.

¹¹ Klarenbeek, A., Recherches expérimentales avec un spirochète, se trouvant spontanément chez le lapin et ressemblant au *Treponema pallidum*, *Ann. Inst. Pasteur*, 1921, xxxv, 326.

region of normal rabbits gave rise, 11 days later, to a slightly excoriated hyperemic reaction without induration, while on the scarified dorsal skin superficial lesions with white crust appeared after 1 to 2 months incubation. The lesions showed spirochetes. Intraocular inoculations in rabbits resulted in some instances in an ulcer on the upper lid and a keratitis after 41 to 43 days; intratesticular inoculations failed to give any spirochetal orchitis.

Spontaneous Instances in America.

The facts reviewed in the foregoing paragraphs suggested the examination of American rabbits. The same condition has been found among the stock rabbits at The Rockefeller Institute. Of 50 rabbits examined in June, 1921, three females and two males were found to have lesions on the vulva, prepuce, and perineum. One (No. 3) of the female rabbits was born at The Rockefeller Institute 6 years ago, has been kept as normal breeding stock ever since, and has never been used for any experimental work.

Recently (November, 1921) six females with similar lesions have been found among twenty rabbits just purchased in Pennsylvania.

Rabbit 1.—Adult female; used for breeding purposes for several months. June 29, 1921. Scaly ulcerative lesions covered with grayish white crusts present on both sides of vulva (Fig. 38). Upon removal of the crusts with forceps, a granular, easily bleeding surface was exposed. The crust and scrapings from the exposed ulcer both revealed the presence in fairly large numbers of a spiral organism resembling *Treponema pallidum* (Figs. 1, 13, and 17). The lesions persisted without much change in extent and character up to the last examination (Sept. 26, 1921). Spirochetes were still numerous. Tissue was removed on June 29, 1921, for inoculation and histological studies. The animal was sacrificed on Sept. 27, and the lymphatic glands were examined for the spirochetes.

Rabbit 2.—Adult nursing female. June 30, 1921. Found to have small, scaly, rather dry lesions on both sides of the vulva; no ulceration. Scrapings examined by dark-field microscope showed in small numbers spirochetes resembling *pallidum*. The lesions remained unchanged up to the last examination (Oct. 13, 1921), but spirochetes were more numerous at that time. The three young rabbits remain entirely free from any lesion, although kept with the mother during a period of 3 months.

Rabbit 3.—Adult female; born at The Rockefeller Institute in 1915 and kept as breeding stock since. June 30, 1921. Scaly, papular elevation or thickening of the vulval region on both sides (Fig. 39), covered by thin grayish crusts over the center of the hyperemic lesions. Examination for spirochetes revealed one fine

type in enormous numbers (Figs. 2 to 4, 14, 15, and 18 to 20) and another decidedly coarser type in smaller numbers (Figs. 5, 6, and 27 to 29). Tissue was excised on June 30, and July 15, 1921, for section and for inoculation. On July 20, 1921, there was no perceptible change in the lesion, and numerous spirochetes were still present (Figs. 9 to 11). This animal was later treated with salvarsan (see below).

Rabbit 4.—Normal adult male; recently purchased. July 6, 1921. Presented small congested and quite swollen but not indurated lesions of the prepuce (Fig. 40), covered with thin crusts; the lesions bled easily when the crusts were removed. The dark-field microscope showed numerous spirochetes similar to those found in the other animals (Figs. 7, 8, 16, and 21 to 24). On July 20, 1921, there were still spirochete-containing lesions. Toward the end of September scaly papular lesions were noticed on nose and lips (Fig. 42), eyelid (Fig. 43), and paws (Fig. 44), and the spirochetes were found to be present there also.

Rabbit 5.—Normal adult male; recently purchased. Sept. 10, 1921. Prepuce swollen and covered with grayish thin scales, under which the tissue was granular, soft, somewhat edematous, and congested (Fig. 41). Numerous spirochetes present. Lesion excised for histological examination.

Spirochetes.

The type of spirochete existing in the lesions of the five rabbits just described closely resembles *Treponema pallidum*. The measurements are: length 10 to 16 μ ; width 0.25 μ . The spiral amplitude, in regular portions, is about 1 to 1.2 μ , and the spiral depth 0.6 to 1 μ ; hence the number of spirals in an individual measuring 10 μ may be about 9 or 10. The last spirals near the extremities are often shallower than those of the middle portion or *vice versa*, and the ends are sharply drawn. A delicate terminal filament has been seen at one, and sometimes at both ends. The shortest specimens measured 7 μ , with 6 spirals, and the longest about 30 μ , with 25 to 28 spirals (Fig. 4). The average is about 12 to 14 μ (Figs. 3, 5, and 6), the organism being slightly longer than the *pallidum* in human lesions. In experimentally inoculated rabbits specimens of *pallidum* as long as 24 μ may be encountered, but the single length is usually 10 to 12 μ (Figs. 12 and 32 to 34). As in the case of *pallidum* (Figs. 35 to 37), specimens with stretched spirals appear in stained preparations (Figs. 25 and 26).

In the lesion of Rabbit 3 there were present two types of spirochete, one the usual fine type and the other decidedly coarser than the average. The morphology of the coarser variety suggests that of

Treponema calligyrum found by the writer in the human condyloma,¹² but it is somewhat longer than *calligyrum* (14 to 18 μ , width 0.35 μ). The spiral amplitude is 1.2 to 1.75 μ and the spiral depth 0.6 to 1.5 μ (Figs. 6 and 27 to 29). This form is perhaps a trace thinner and longer than the *calligyrum*, or medium type smegma treponema of the normal human genitalia. The organism rotates actively, with occasional bending at the middle, but no vibratory or lashing movements have been observed.

The rabbit treponema forms entangled masses of long threads, 30 μ at least (Figs. 4 and 11), a phenomenon I do not recall having seen in the case of *Treponema pallidum*. The agglomeration of organisms in a striking stellate mass of several to a dozen individuals is frequently observed (Fig. 10). A similar behavior has been observed in young, actively motile, pure cultures of *Treponema pallidum*. Both *pallidum* and the rabbit treponema sometimes form a ring by touching one end with the other while actively rotating, and occasionally they make a knot of themselves. In a film preparation from the lesions of Rabbit 3, stained by Fontana's method, occasional wavy, but not spiral forms were seen; perhaps they were degenerated or involution forms (Figs. 30 and 31).

The staining properties of these rabbit spirochetes are the same as those of *Treponema pallidum*. Both can be readily stained by ordinary basic aniline dyes when fixed in a buffered formaldehyde solution, as shown by Miss Tilden.¹³ Jacobsthal proposed for the organism the name *Spirochaeta paraluis cuniculi*, but in view of its close resemblance to *Treponema pallidum* and *Treponema pertenue* it may perhaps be more appropriate to use the simpler name *Treponema cuniculi*.

Examinations of smegma and mucous secretions on the genitalia of many normal male and female rabbits failed to reveal any spirochetal organisms, which circumstance does not of course exclude the possibility that spirochetes are occasionally present in such materials.

¹² Noguchi, H., Cultivation of *Treponema calligyrum* (new species) from condylomata of man, *J. Exp. Med.*, 1913, xvii, 89.

¹³ Noguchi, H., A note on the venereal spirochetosis of rabbits. A new technic for staining *Treponema pallidum*, *J. Am. Med. Assn.*, 1921, lxxvii, 2052.

Histology of the Lesions.

Excised tissue from the undisturbed spontaneous lesion on the prepuce of Rabbit 5 (Fig. 41) was fixed in Zenker's fluid and sections were stained with hematoxylin-eosin and eosin-methylene blue. The epidermis was thickened and the papillæ were large and prominent; the infiltration was spotty and mainly confined to the interpapillary layers. The new cells were mainly mononuclear, partly eosinophils, and few or no polymorphonuclears were present. In general features the lesion suggests a condyloma. Although certain blood vessels were surrounded by mononuclear cells, no definite endothelial proliferation was apparent (Fig. 48). The lesion as described may be taken as the pure type before secondary bacterial infection has come in to induce acute inflammation and mar the picture. Once ulceration has taken place, as in the vulval lesions of Rabbits 1 and 3 (Figs. 38 and 39), the interpapillary infiltration of mononuclear cells is accompanied by numerous polymorphonuclear leucocytes, which invade also the stratified layers of the thickened epidermis. Minute hemorrhages and necrotic foci are then occasionally met with.

For comparison with these lesions, a scrotal chancre was induced with the Nichols strain of *Treponema pallidum* long adapted to the rabbit (since 1912). The chancre on the 28th day was indurated and stood out as an oval mass from the surrounding loose connective tissue (Fig. 47); in histology it differed widely from the genital lesions induced by the rabbit spirochete. The corium was highly infiltrated with mononuclear cells, some showing eccentric nuclei and checker-board chromatin. Polymorphonuclear leucocytes were absent. But the striking difference was in the blood vessels, which were surrounded by the new cells (Fig. 49).

Repeated attempts to stain the spiral organism in the tissue by means of silver impregnation have been only partially successful. The organisms near the surface of the lesion took the stain. By careful teasing of the deeper layers and dark-field examination, spirochetes have been found to be present in all parts of the lesions.

Transmission Experiments.

First Passages.—Sixteen rabbits and four monkeys¹⁴ (*Macacus rhesus*) were inoculated with the material containing the spirochetes, usually on the scarified prepuce or vulva. In rabbits some intratesticular, intracorneal, and intraocular inoculations were made; in monkeys the scarified eyebrows, as well as prepuce or vulva, were smeared with the scraping of the rabbit lesion. Each of the original rabbits was paired in a separate cage with a normal rabbit of opposite sex for the purpose of sexual transmission.

Reproduction of the disease on the vulva or prepuce respectively was obtained in all five of the female and in eight of the eleven male rabbits inoculated (Table I). One strain (Rabbit 3) produced in two male rabbits a superficial scaly, papular lesion on the scrotal skin at the site of testicular inoculation (Fig. 45), and in one of them a lesion developed also on the prepuce. The results of intratesticular inoculation were in general unsatisfactory. In most cases, owing to the impure nature of the material, a more or less acute suppurative orchitis ensued, and no spirochetes were present in these lesions. Negative results also followed intraocular and intracorneal inoculations and the application of the material to the scarified surface of the cornea. The lymph glands were not noticeably enlarged either in the original rabbits or in those inoculated. The inoculation of emulsions of popliteal and inguinal lymph glands from ten rabbits infected with *Treponema cuniculi* into both testicles of four normal male rabbits did not produce any orchitis within 3 months. On the other hand, similar emulsions from rabbits infected with *Treponema pallidum* gave rise to typical orchitis within 1 month.

¹⁴ Four additional monkeys were later inoculated with different strains of *Treponema cuniculi*, but none showed any lesion during a period of observation of 3 months. In order to determine whether the adaptation to rabbits of the human strain of *Treponema pallidum* had rendered it avirulent for monkeys, two monkeys were inoculated with the Nichols strain (1912). Both presented typical lesions within 1 month. Two monkeys inoculated from a case of human chancre showed typical lesions within 2 months. The pathogenicity of *Treponema cuniculi* appears, therefore, to be different from that of *Treponema pallidum* of human syphilis.

TABLE I.
Transmission Experiments.

Animal No.	Sex.	Source of inoculated material.	Site of inoculation.	Date and mode of inoculation.	Results.	Period of incubation.	Remarks.
Rabbits.						days	
6	Female.	Rabbit 1	Vulval mucosa.	June 30, 1921. Scarification and smearing.	Scaly lesion at site of inoculation first noticed Sept. 26, 1921.	88	
7	"	" 1	"	"	Definite lesion Sept. 13, 1921.	75	
8	Male.	" 1	Prepuce.	"	Ulcer with thin crust on prepuce Aug. 10, 1921.	41	
9	"	" 1	"	"	No lesion after 90 days.		
10	"	" 1	"	"	Definite scaly lesion around anus Sept. 26, 1921. No lesion after 90 days.	88	
			Right cornea.	"	No orchitis after 90 days.		
			" testicle.	June 30, 1921. 1 drop intratesticularly.			
11	"	" 1	Prepuce.	June 30, 1921. Scarification.	Definite lesion Sept. 26, 1921.	88	
			Right cornea.	"	No lesion after 90 days.		

11	Male.	Rabbit 1	Right testicle.	June 30, 1921. 1 drop intratesticularly.	Abscess formation and induration lasting for 1 mo. No spirochetes found in testicular puncture.	
<i>Macacus rhesus.</i> 1	"	" 1	Prepuce. Left eyebrow.	June 30, 1921. Scarification. "	No lesion after 80 days. "	Reinoculated with material from Rabbit 4, Sept. 19, 1921.
		" 1	Prepuce. Left eyebrow.	" "	" "	
		" 1	Prepuce. Left eyebrow.	" "	" "	
Rabbits. 12	Female.	" 2	Vulva.	July 1, 1921. Scarification.	Scaly lesion Sept. 13, 1921, since spread.	75
13	"	" 2	"	"	Small lesion July 20, 1921, now slightly increased.	20
14	Male.	" 2	Prepuce. Cornea. Right testicle.	" " July 1, 1921. Intratesticular.	Definite lesion Aug. 17, 1921. None. Abscess formation in 7 days, extended to left testicle.	47

TABLE I—Concluded.

Animal No.	Sex.	Source of inoculated material.	Site of inoculation.	Date and mode of inoculation.	Results.	Period of incubation.	Remarks.
Rabbits. 15	Male.	Rabbit 2	Prepuce.	July 1, 1921. Scarification.	Definite lesion Sept. 26, 1921.	days 88	
			Cornea. Right testicle.	" July 1, 1921. Intratesticular.	None. Suppurative lesions after 1 wk., remained firm for 40 days.		
<i>Macacus rhesus</i> . 3	"	"	Prepuce.	July 1, 1921. Scarification.	None.		
			Right eye-brow.	"	"		
4	Female.	"	Vulva.	"	"		
			Eyebrow.	"	"		
Rabbits. 16	"	"	Vulva.	July 2, 1921. Scarification.	Definite lesion Aug. 16, 1921.	45	
17	Male.	"	Prepuce.	"	Definite lesion Aug. 17, 1921.	46	
			Both testicles.	July 2, 1921. Intratesticular.	Scab at lower part of left scrotum Aug. 17, 1921. Lesion slightly indurated and many spirochetes found.	46	
18	"	"	Prepuce.	July 2, 1921. Scarification.	No lesion developed.		

18	Male.	Rabbit 3	Right testicle.	July 2, 1921. Intra-testicular.	Abscess formation. Scaly lesions on left scrotum Aug. 20, 1921. Spirochetes+.	49	Photographed (Fig. 45) Sept. 13, 1921 (73 days).
19	"	" 3	Prepuce.	July 5, 1921. Scarification.	Definite lesion Sept. 13, 1921.	70	
<i>Macacus rhesus.</i> 3	"	" 3	Left testicle.	July 5, 1921. Intra-testicular.	No lesion developed.		
			" eyebrow.	July 2, 1921. Scarification.	" " "		Right eyebrow had been inoculated from Rabbit 3 on July 1, 1921.
Rabbits. 20	"	" 4	Prepuce.	July 5, 1921. Scarification.	Definite lesion Aug. 10, 1921.	36	
			Cornea.	July 2, 1921. Intra-corneally with syringe.	Opacity still persists after 85 days.		
			Both testicles.	July 2, 1921. Intra-testicular.	Two small nodules on left testicle (no spirochetes).		
21	"	" 4	Prepuce.	July 5, 1921. Scarification.			Died of septicemia in 8 days.
			Eye.	July 5, 1921. Intraocular (anterior chamber).			
			Both testicles.	July 5, 1921. Intratesticular.			
Average.....						60½	

The period of incubation varied from 20 to 88 days, the average being $60\frac{1}{2}$ days.

Subsequent Passages.—All five strains are being maintained in rabbits. The strain from Rabbit 1 has now passed through four transfers: first, June 30, 1921, positive September 13 (75 days); second, September 27, positive October 17 (20 days); third, October 17, positive October 22 (5 days); fourth, October 24, positive November 10 (17 days).

Inoculations of monkeys have remained up to the present (4 months) without result. One of the monkeys was inoculated with two different strains, on right and left eyebrow respectively. Two other monkeys have recently been reinoculated in the eyebrow, and also into the hypogastric region, with a hypodermic needle, the infective material being deposited intradermally.

Sexual Transmission.—Rabbit 1 was kept in the same cage with three males successively (July 12 to September 26), Rabbit 2 with two males, and Rabbit 3 with one male (July 12 to October 15). Rabbit 22, 3 months after having been placed with Rabbit 3, showed a small papular lesion on the prepuce in which spirochetes were numerous.

Attempts to Produce Chancre on the Scrotum.

Treponema pallidum from human syphilis is capable of producing chancre-like lesions on the scrotum of rabbits when a small piece of *pallidum*-containing tissue is subcutaneously introduced (Tomasczewski,¹⁵ Uhlenhuth and Mulzer,⁶ Brown and Pearce,¹⁶ and others). To determine whether or not the rabbit spirochete is capable of producing a similar lesion in rabbits, portions of the erosive, spirochete-containing lesion from the vulvolabial region of Rabbit 3 were introduced into the subcutaneous pocket of each of the scrota of two adult male rabbits, two other adult males being simultaneously inoculated with fragments of freshly excised scrotal chancre from a syphilitic rabbit (Nichols strain).

¹⁵ Tomasczewski, Ueber eine einfache Methode, bei Kaninchen Primäraffekte zu erzeugen, *Deutsch. med. Woch.*, 1910, xxxvi, 1025.

¹⁶ Brown, W. H., and Pearce, L., Experimental syphilis in the rabbit. II. Primary infection in the scrotum. Part 1. Reaction to infection, *J. Exp. Med.*, 1920, xxxi, 709.

One of the rabbits (No. 23) inoculated with the rabbit spirochete showed at one site of insertion after 27 days a small crust under which the surface was soft, pale, and granular, but there was no marked induration. Spirochetes were not found in the lesion until 45 days after inoculation. The other site remained small and reddish, with a trace of infiltration, but no spirochetes were found. The other rabbit (No. 24) showed slight infiltration at one site of insertion (Fig. 46), but no definite, chancre-like induration developed during 2 months observation. Brownish scaly patches rich in spirochetes appeared on the right scrotum after 45 days.

Of the two syphilitic controls, Rabbit 25 developed within 3 weeks at the site of insertion on the right scrotum a typical chancre which attained a diameter of 2 cm. in 28 days (Fig. 47). The second control (Rabbit 26) had not developed a chancre 75 days after inoculation. Two rabbits inoculated with chancre material from Rabbit 25 developed typical chancres within 3 weeks, while simultaneous inoculations from the slightly indurated (*cuniculi*) lesion of Rabbit 23 were unsuccessful.

It is possible that a chancre may yet be produced with the rabbit spirochete by inoculation of a large number of animals. The number of spirochetes in the *pallidum* chancre of rabbits is never as large as that found in the *cuniculi* lesions in rabbits.

For the purpose of comparison, *pallidum*-containing material was applied to the scarified prepuce or vulva of rabbits and gave rise to chancre-like nodules. The appearance of these lesions, however, was entirely different from that of the lesions produced by *Treponema cuniculi*.

Typical *cuniculi* lesions may be produced on the scrotal skin or prepuce of rabbits having active *pallidum* lesions, and the reverse is also true. The infections appear to follow independent courses.

Wassermann Reaction.

The sera of the eleven rabbits with spontaneous lesions, and of thirty-three experimentally inoculated from them, have been tested for the Wassermann reaction. The acetone-insoluble lipoids served as antigen, and the inactivated sera were used in quantities of 0.1 and 0.2 cc. Twenty-four of the animals had active, spirochete-

containing lesions of the genital region at the time of test; twenty had recently been inoculated and had not developed any lesions. The results of the tests were uniformly negative.

The sera of four rabbits having active scrotal chancres (Nichols strain of *Treponema pallidum*) were simultaneously tested; three gave a strongly positive (++++), and the other a partial (+) reaction. Twelve rabbits which had developed chancres 2 months after inoculation on vulva or prepuce with *pallidum* gave strongly positive (++++) reactions.

Thus far positive Wassermann reactions have been obtained only with the sera of rabbits having active syphilitic lesions. Apparently no complement-fixing substance, such as exists in the blood of syphilitic human beings or experimentally infected syphilitic rabbits, was present in the blood of animals which had been infected with the rabbit treponema for periods varying from 1 or 2 weeks to many months. Whether or not the fixing substance will eventually appear in the sera of these rabbits remains to be seen.

Effect of Salvarsan.

Rabbit 3 was given intravenously on August 17, 1921, 0.02 gm. of salvarsan per kilo of body weight. Rabbit 27, having a chancre of thumb size on each scrotum, was similarly treated at the same time to serve as control.

Rabbit 3.—Aug. 17, 1921. Showed papuloedematous, partially eroded, moist lesion, extending over the entire vulval region, including the labial mucosa and adjacent skin; perineum also scaly, congested, and swollen. Dark-field examination revealed spirochetes in large numbers. 5 p.m. 4.4 cc. of a 1:10 dilution of slightly alkaline salvarsan solution was intravenously administered (0.02 gm. per 1,000 gm. of body weight). Aug. 18, 5 p.m. Considerable reduction in swelling, less irritation, apparent drying up of lesion; no spirochetes found. Aug. 20. Lesion near hair line of vulva still somewhat moist and crusted, inferior portion practically healed; anal lesion still present. Spirochetes were no longer found in any of the suspicious spots. Aug. 26. Only small scabs present. Sept. 13. Slight scaliness at hair line of vulva; no spirochetes.

Rabbit 27.—Aug. 17, 1921. Chancre (human strain isolated by Nichols in 1912, since maintained in rabbits) on each scrotum, 2 cm. in diameter; cartilaginous induration, with small umbilicated center covered by dark dry crust. Many

spirochetes present. 5 p.m. 5 cc. of a 1:10 dilution of slightly alkaline salvarsan (0.02 gm. per 1,000 gm. of body weight) intravenously administered. Aug. 18. Lesions softer, smaller; no spirochetes. Aug. 20. Lesions soft. Aug. 23. Lesions have almost disappeared. Sept. 13. No trace of lesions found.

The experiment indicates that the *cuniculi* lesions and the experimental scrotal chancre caused by *Treponema pallidum* from human syphilis are similarly influenced by salvarsan. In both instances the spirochetes disappeared within 24 hours after the administration of the drug, and the lesions themselves within 9 days. No recrudescence has occurred.

DISCUSSION AND SUMMARY.

Of 50 rabbits, otherwise regarded as normal, three adult females and two adult males (10 per cent) have been found to have in their genitoperineal region certain papulosquamous, often ulcerating, lesions. A recently purchased group of twenty rabbits contained six females (30 per cent) with similar lesions. This condition runs a chronic course and is characterized by the presence of a spiral organism closely resembling *Treponema pallidum*.

The rabbit spirochete has the same morphological features as *Treponema pallidum*; it is possibly a trifle thicker and longer than the average *pallidum*. Long specimens measuring 30 μ are frequently encountered, and they show a tendency to form loosely entangled knots. A stellate arrangement of several organisms in a mass is frequently observed.

In the lesion of one rabbit there were two types of spirochete, one of the variety just described, the other a somewhat coarser organism, closely resembling *Treponema calligyrum* found in a human condyloma, but a trifle thinner and longer. This organism is perhaps merely a variant type of the rabbit spirochete.

The histological reactions are similar to, but considerably less cellular, than those occurring in typical primary syphilitic lesions. There is a marked hyperkeratosis and interpapillary infiltration not observed in scrotal chancre.

The disease is transmissible to normal rabbits, in which the usual papular lesions can be readily reproduced in the genitoperineal region. In the first passages the incubation period varied from 20 to

88 days; subsequently one of the strains produced a lesion in 20 days on the second, and in 5 days on the third passage. No typical orchitis or keratitis was produced in the rabbits of the present series, although in one of the original rabbits (No. 4) scaly, papular lesions have developed on the nose, lips, eyelid, and paws. Monkeys (*Macacus rhesus*) failed to show any lesions within a period of 4 months after inoculation.

In one instance transmission was accomplished through the mating of an infected female with a normal male.

The Wassermann reaction was uniformly negative in the five rabbits with spontaneous lesions and in eighteen rabbits experimentally infected.

Salvarsan had the same therapeutic effect on the lesions produced by the rabbit spirochete as on the experimental *pallidum* lesion of the rabbit.

The organism belongs to the genus *Treponema*, and may be designated *Treponema cuniculi*.

EXPLANATION OF PLATES.

PLATE 17.

Dark-field photographs. Magnification $\times 1,000$.

FIG. 1. *Treponema cuniculi* from Rabbit 1.

FIGS. 2 to 5. *Treponema cuniculi* from Rabbit 3. Fig. 4 shows one of the very long specimens.

FIG. 6. The coarser type of treponema from Rabbit 3.

FIGS. 7 and 8. *Treponema cuniculi* from Rabbit 4.

FIGS. 9 to 11. *Treponema cuniculi* from Rabbit 3. Fig. 11 shows an entangled mass of the long forms. Fig. 10 illustrates the agglomeration of organisms in stellate form which is frequently seen.

FIG. 12. *Treponema pallidum* from experimental syphilitic orchitis in the rabbit.

PLATE 18.

Magnification $\times 1,000$.

FIG. 13. Film preparation of exudate from Rabbit 1, stained with Giemsa solution.

FIGS. 14 and 15. Film preparation of exudate from Rabbit 3, stained with Giemsa solution.

FIG. 16. Film preparation of exudate from Rabbit 4; Giemsa stain.

FIG. 17. Film preparation from Rabbit 1, stained by Fontana's method.

FIGS. 18 to 20. Film preparation from Rabbit 3, stained by Fontana's method.

FIGS. 21 to 24. Film preparation from Rabbit 4, stained by Fontana's method.

FIGS. 25 and 26. Irregular forms of *Treponema cuniculi* from Rabbit 3, stained by Fontana's method.

FIGS. 27 to 29. Spirochetes resembling *Treponema calligyrum* from Rabbit 3, stained by Fontana's method.

FIGS. 30 and 31. Non-spiral forms from Rabbit 3.

FIGS. 32 and 33. *Treponema pallidum* in rabbit orchitis, stained by Fontana's method.

FIG. 34. *Treponema pallidum* in rabbit orchitis. Mordant Gentian violet.

FIGS. 35 to 37. Irregular forms of *Treponema pallidum* from experimental scrotal chancre, stained by Fontana's method.

PLATE 19.

FIGS. 38 to 41. Typical lesions (from Rabbits 1, 3, 4, and 5 respectively) on the rabbit genitalia.

FIG. 42. Lesions in Rabbit 4 on the nose and left upper lip.

FIG. 43. The same, on the left eyelid.

FIG. 44. The same, on the left paw near the joints of the nails.

FIG. 45. An erosive lesion at the site of intratesticular inoculation in Rabbit 18 (transfer from No. 3) after an incubation period of 49 days. There was only a slight induration, but numerous spirochetes were present.

FIG. 46. The healing scar in Rabbit 24 at the site of pocket insertion of the infective material in the unsuccessful attempt to produce chancre (photograph taken 28 days after inoculation). *Treponema cuniculi* found after 73 days.

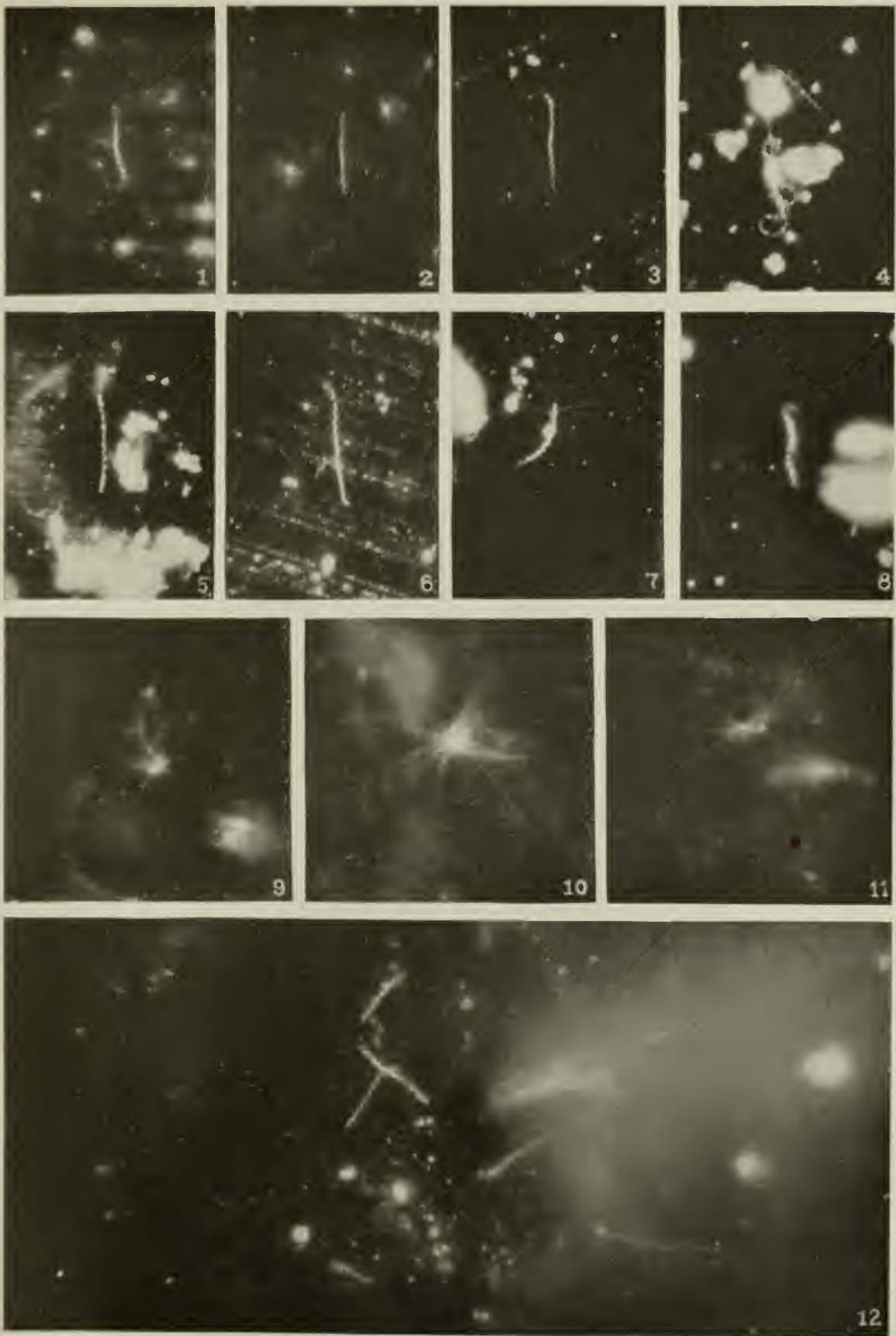
FIG. 47. A typical chancre, for comparison, on the scrotum of Rabbit 25, inoculated with the Nichols strain of *Treponema pallidum*. Note the cartilaginous, well circumscribed, umbilicated chancre (photographed 28 days after insertion).

PLATE 20.

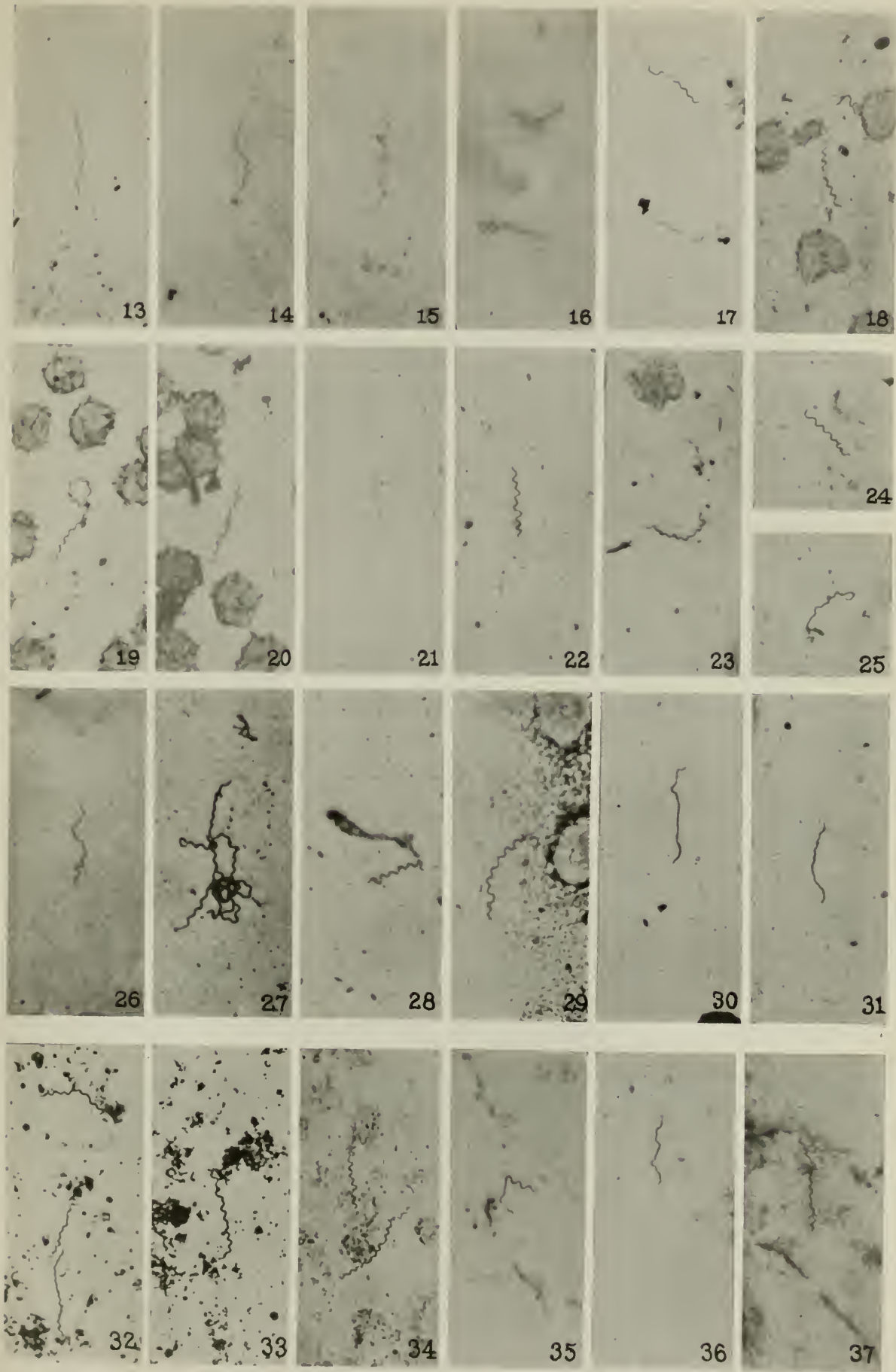
FIG. 48. Histological changes of the lesion on the prepuce of Rabbit 5. Zenker fixation, stained by hematoxylin-eosin. Showing the marked hyperkeratosis and considerable mononuclear infiltration of the interpapillary spaces. $\times 75$.

FIG. 49. Section of chancre on scrotal skin of Rabbit 25 (Nichols strain) 28 days after inoculation, showing typical interstitial and perivascular infiltration of mononuclear cells, and almost normal epidermis. The same fixation and staining as Fig. 48, shown for comparison. $\times 75$.

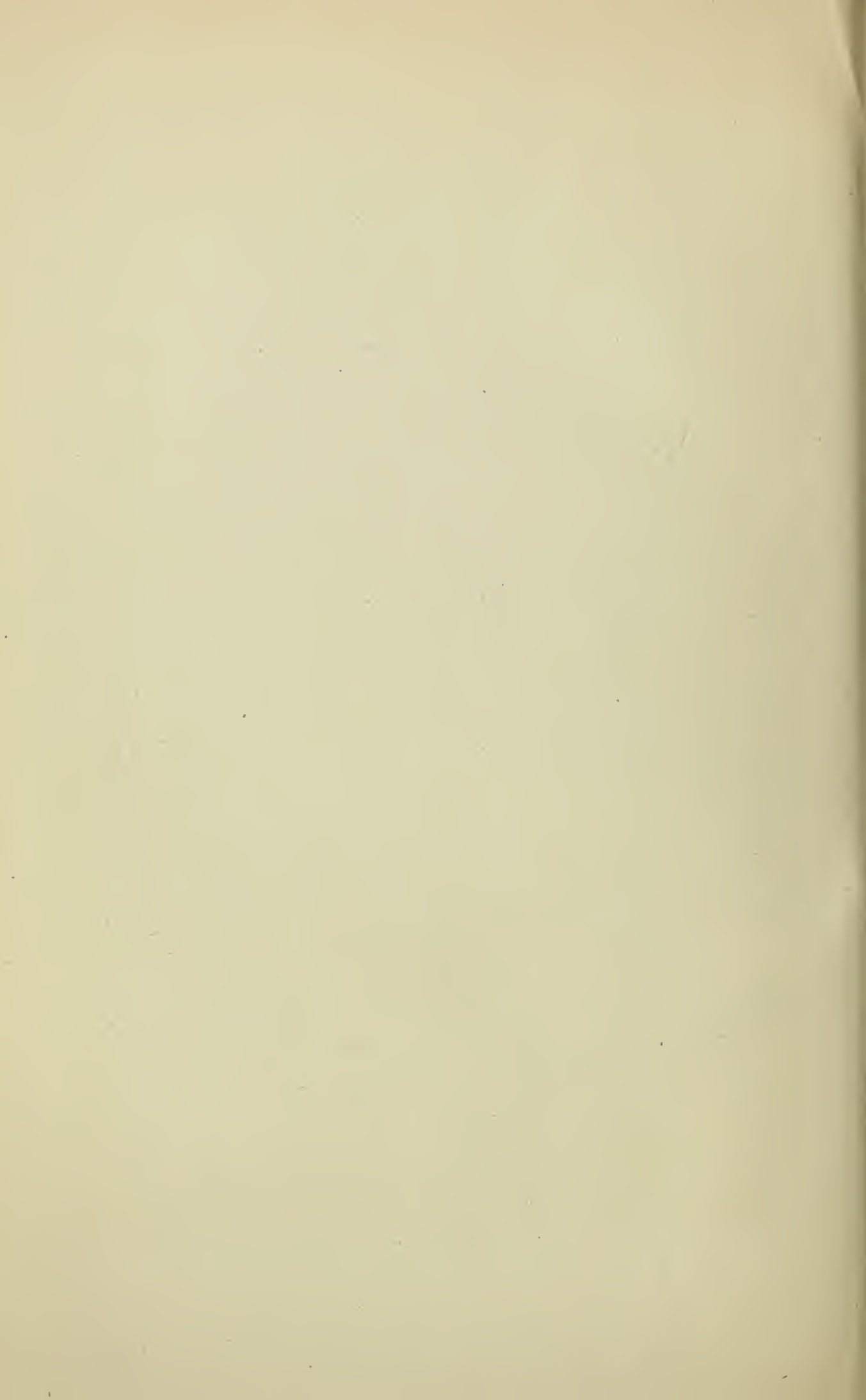
4081



(Noguchi: Venereal spirochetosis in American rabbits.)



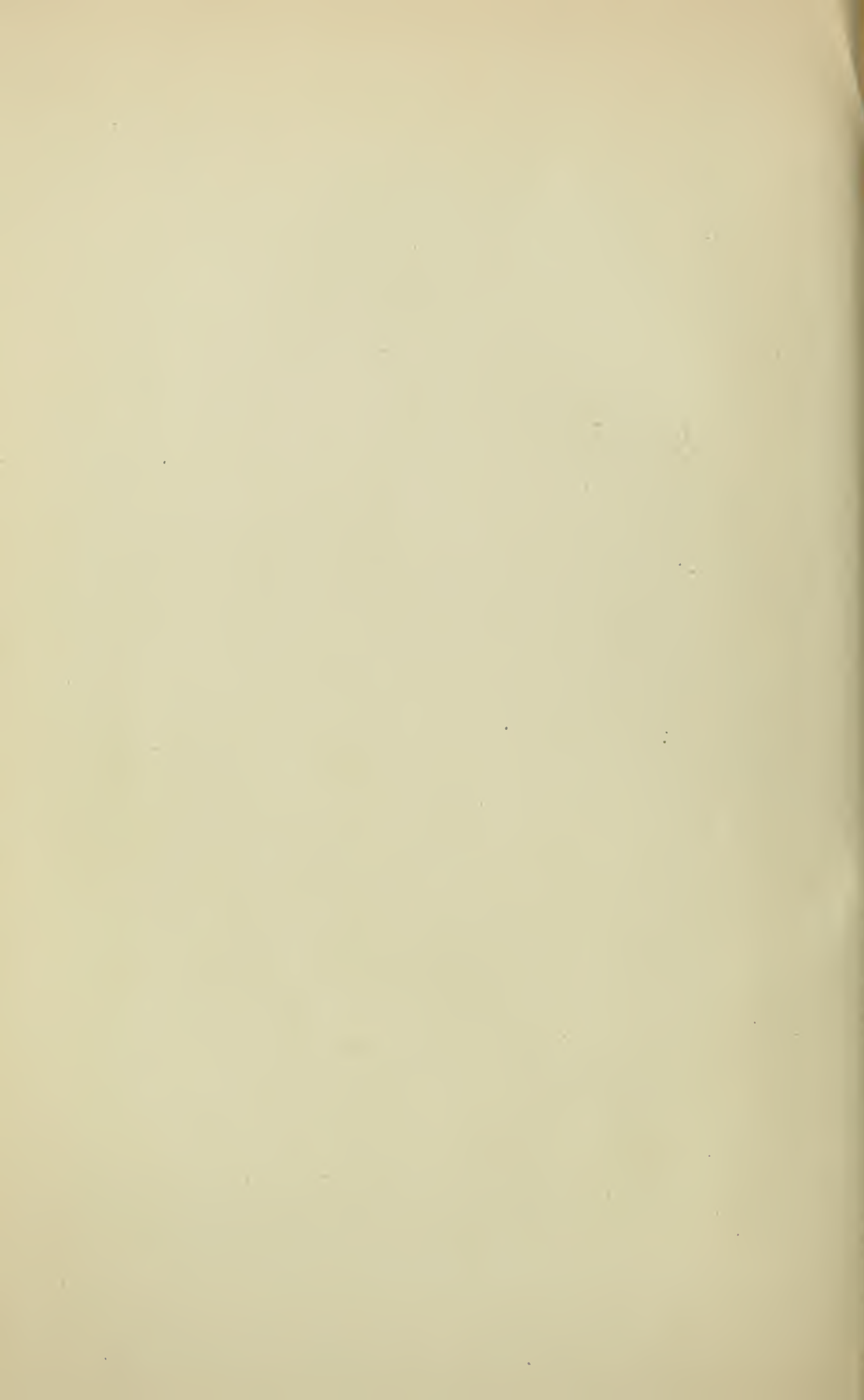
(Noguchi: Venereal spirochetosis in American rabbits.)

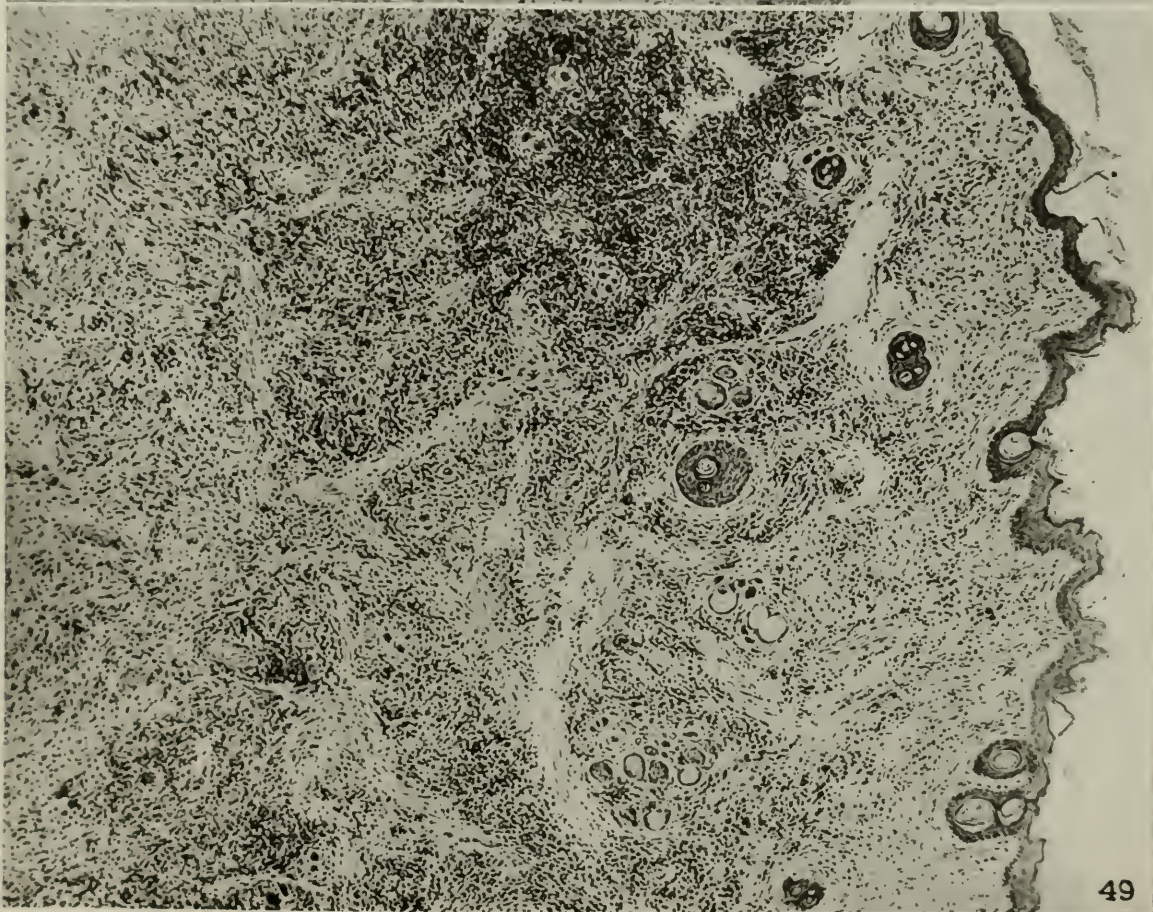
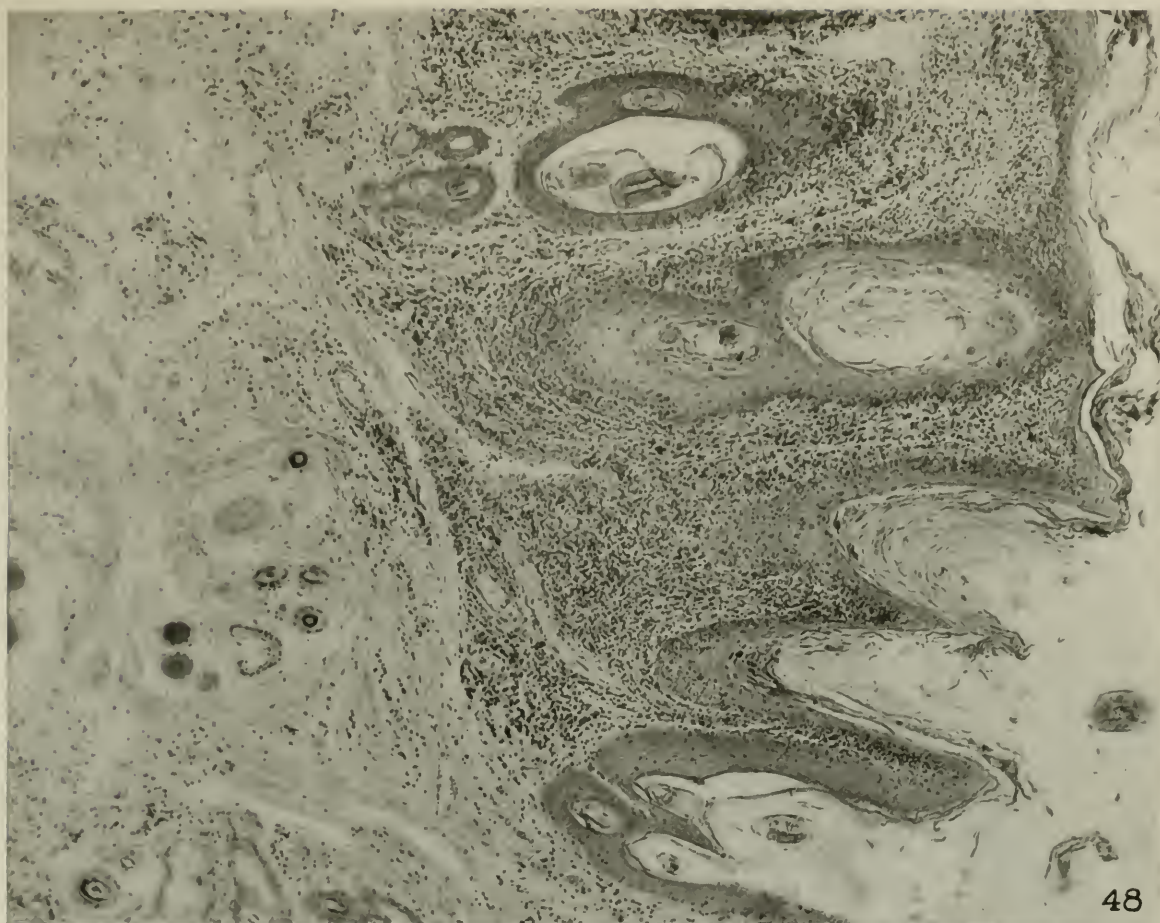


408³



(Noguchi: Venereal spirochetosis in American rabbits.)





(Noguchi: Venereal spirochetosis in American rabbits.)



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HEMOLYTIC ACTION OF A STAPHYLOCOCCUS DUE TO A FAT-SPLITTING ENZYME.

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In the routine examination of a sample of milk two dilutions plated in blood agar showed different effects. The plate of a 1:100 dilution indicated a pure culture of a non-hemolytic staphylococcus, while the plate of a 1:10 dilution appeared to be a pure culture of a hemolytic staphylococcus. In the latter case the deep as well as the surface colonies showed clear hemolytic zones with a few corpuscles remaining next to the colony. This hemolysis is unusual for a staphylococcus which, ordinarily, even when hemolytic on the surface does not give evidence of hemolysis around the deep colonies. A culture was prepared by inoculation from a single deep hemolytic colony. The platings were repeated with this culture, and the same results obtained. These observations indicated that some constituent of the medium was responsible and not the presence of both a hemolytic and a non-hemolytic organism. The following study was, therefore, made to determine more accurately the precise factors involved.

The relation of the milk to the hemolytic process was outlined by plating the culture on (a) plain blood agar, (b) blood agar plus 0.1 cc. of sterile fat-free milk, (c) blood agar plus 0.1 cc. of whole milk, and (d) blood agar plus 0.02 cc. of cream. The results given in Table I show that hemolysis did not occur around the deep colonies in either the plain blood agar or in that to which fat-free milk had been added, but it did occur in the plates made with whole milk and with cream. Similar results occurred when bouillon was used instead of agar. They indicated that the effect of the dilution noted originally corresponded to a lowered fat content. The problem, therefore, resolved itself into a question of the relation of the fat to hemolysis and the action of the organism on the fat.

Characteristics of the Organism from Milk, Designated Staphylococcus A.—The organism is a small Gram-positive coccus, occurring often in pairs and irregular masses, and frequently in groups of three. On an agar slant the growth is smooth, opaque, and slightly yellowish white. On a blood agar plate the surface colonies are round, opaque, smooth, and more distinctly yellowish in color. In plain bouillon there is a good clouding, the growth adheres to the sides of the tube often forming a ring at the surface, and there is a small amount of sediment. Gelatin is liquefied after about 2 weeks. Milk is coagulated in 5 to 15 days. Acid is formed in glucose, lactose, sucrose, maltose, and mannitol bouillon, but no gas is produced. All these characteristics identify the organism as a staphylococcus.

TABLE I.

Hemolytic Action of Staphylococci in Media Containing Defibrinated Horse Blood with and without Cream.

	Control without milk.	Fat-free milk. 0.01 cc.	Whole milk 0.01 cc.	Cream 0.002 cc.
Blood agar plus culture (plated).....	—*	—	+	+
“ bouillon plus culture.....	—	—		+++
“ “	—	—		—

In the case of the agar the results are given for the deep colonies only. The quantities of milk, etc., indicated are those added to 1 cc. of media.

* In all the tables + indicates hemolysis; — no hemolysis.

Preparation of Blood.—The blood corpuscles obtained from horse blood were washed with 0.85 per cent salt solution and suspended in this fluid made up to the original volume of the defibrinated blood. It was added in the proportion of 1 drop to a cc. of bouillon. Whole defibrinated horse blood was used in the agar plates, about 1 cc. to each plate.

Standard agar was used for the plates in 10 to 12 cc. amounts. Standard bouillon was used for the bouillon cultures. 1 cc. of bouillon was measured into each tube. 0.02 cc. of 15 per cent cream diluted to 1:10 and 1 drop of blood were added to each cc. All tubes were incubated at 37°C. over night.

The results of the experiments are contained in Tables I to VI. The nature of the hemolytic zone on the blood agar plates suggested that the active hemolytic agent diffused out from the colonies; *i.e.*, that the process was extracellular since the hemolytic zone extended for a considerable distance beyond the colony. To determine the presence of an extracellular hemolytic agent the experiments summarized in Table II were carried out.

The staphylococci were killed and any extracellular enzymes present left unharmed. Such a procedure excluded the use of heat. We adopted ether, and later chloroform for control work, as an agent which would destroy the staphylococcus and which could then be removed from the media and thus would not interfere with the subsequent experiments because of its own hemolytic action. The

TABLE II.

Hemolytic Action of Bouillon Cultures of Staphylococci Sterilized with Ether or Chloroform to Which Horse Blood Corpuscles and Milk Fat Were Added.

	Cream (15 per cent) 0.002 cc.	Fat-free milk 0.01 cc.	Control without fat.
Etherized bouillon culture plus corpuscles.....	+++	—	—
Chloroform “ “ “ “	+++	—	—

preparation of these etherized cultures was as follows: The organism was grown in standard bouillon 24 to 48 hours. Ether was added and the culture fluid shaken and allowed to stand at room temperature. The next day the sterility was tested by inoculating a tube of bouillon with 2 or 3 drops of the etherized culture. The ether was driven off by blowing air into the tube through a sterile pipette plugged with cotton. This procedure was repeated three or four times by alternately warming the culture in the incubator and then blowing air through it while cooling. This drove out practically all ether. That the specific effect of the ether was eliminated was indicated by the control tests. The results obtained with the etherized cultures, and confirmed with cultures treated with chloroform, show that after the staphylococcus has grown in bouillon the agent destroying the living organism does not destroy the substance which, in conjunction with

the cream or fat, produces hemolysis of red blood corpuscles. In the absence of cream or a fat hemolysis fails to occur.

It seemed evident that we were dealing with a lipolytic enzyme. In such a case if sufficient heat be applied either to the living culture or to the killed culture hemolysis should be prevented. The data indicating the effect of heat are summarized in Table III.

TABLE III.
Hemolytic Action of Both Living and Killed Bouillon Cultures of the Staphylococcus Which Had Been Heated before the Cream and Blood Corpuscles Were Added.

	Temperature.	Time.	Hemolytic action.
	°C.	min.	
Living culture.....	Unheated.		+++
	45	30	++*
	65	30	—
	100	5	—
Culture killed by ether.....	Unheated.		++
	45	30	++
	55	30	++
	65	30	—
	100	5	—
“ “ “ chloroform.....	Unheated.		+++
	45	30	+++
	55	30	+++
	65	30	—
	100	5	—

Final concentration of cream was 0.002 cc. per cc. of culture fluid.

* After heating to 45°C. the culture continued to grow.

It appeared at first that hemolytic action in the etherized culture was destroyed when the fluids were heated to 45°C. Later tests showed that the first results were due to the fact that the fat added to the tube after heating rose to the surface and was, therefore, acted upon very slowly. By occasionally shaking the tubes hemolysis occurred in tubes heated at 45°C. and also at 55°C., but at 65°C. hemolytic action was destroyed.

Having found that the ultimate hemolytic process was extracellular and thermolabile the next step was to eliminate the enzyme from the

final stage of hemolysis. This was done by allowing the living staphylococcus or the etherized culture to act upon cream or fat and then to destroy the organism or enzyme with heat before adding blood corpuscles. It was found that when the living culture or the etherized culture fluid was heated to 100°C. for 5 minutes neither of the fluids thus treated would hemolyze red blood corpuscles. On the other hand, when the living culture was in contact with cream for 4 hours and the etherized culture with cream for 18 hours and these fluids were then heated to 100°C. for 5 minutes hemolysis resulted. Success with this phase of the problem was no doubt due to the absence of complex protein material in the bouillon. To demonstrate this fact small amounts of serum were added to previously heated tubes of bouillon which were hemolytically active, and the resulting mixture was heated to 100°C. The addition of 1 or 2 drops of serum to 1 cc. of bouillon had no effect upon the hemolytic action of the bouillon; the addition of 4 drops resulted in a slight hemolysis, while the addition of 7 drops of serum resulted in a failure of the heated culture fluid to hemolyze the red blood corpuscles.

The substrate for the action of the lipase in the previous experiments was cream. To eliminate cream as a factor *per se*, other fats were tested with the staphylococcus and with the etherized culture fluid, such as butter, olive oil, nut butter, triolein, triacetin, tributyrin, and pork fat. The results are contained in Table IV.

Preparation of Fats.—Preliminary experiments suggested that a high degree of emulsification of fat was desirable. Soaps of certain fatty acids will lysis red blood cells, consequently emulsification with an alkali was not feasible. Fair results were obtained by shaking various fats or oils with bouillon. The quantity of fat used was such that the concentration would approximate the quantity of fat added to the culture when 15 per cent cream was used.

Cream was drawn from the top of a tube of milk after it had stood several hours. This was sterilized fractionally in an Arnold sterilizer at 100°C. for 20 minutes on 3 successive days. For use it was diluted to 1:10 with sterile bouillon, making approximately a 1.5 per cent suspension. Fat-free milk was obtained by centrifuging and lifting off the cream with a spatula. This was sterilized fractionally. A suspension of olive oil was made by adding 1.5 cc. to 10 cc. of bouillon

and shaking in a mechanical shaker 1 to 2 hours. Suspensions of other fats, such as butter, nut butter, triacetin, triolein, tributyrin, and pork fat, were made in a similar way in bouillon and shaken. The final concentration of the fats in the experimental tubes was approximately 0.002 cc. per cc. of bouillon.

Positive results were obtained with emulsions of butter, olive oil, and triolein. With some fats the hemolytic action was less marked than with others. The difference in the action seems to be due, in part at least, to the character of the emulsion, for with those fats which were associated with hemolysis the more highly emulsified they were the better the results; furthermore, a shaken tube would often give

TABLE IV.

Hemolytic Action of Living and Etherized Bouillon Cultures of the Staphylococcus to Which Were Added Horse Blood Corpuscles and Various Fats.

	Cream.	Butter.	Olive oil.	Tributyrin.	Triolein.	Triacetin.	Nut butter.	Pork fat.	Fat-free milk.	Control without fat.
Living bouillon culture plus corpuscles.....	+++	+	++	-	++	-	-	-	-	-
Etherized bouillon culture plus corpuscles.....	++	+	+		+		-	-	-	-

Final concentration of fat was approximately 0.002 cc. per cc. of bouillon.

a greater degree of hemolysis than an unshaken tube. There is considerable evidence in the literature to the effect that the salts of unsaturated fatty acids hemolyze red blood corpuscles more readily than the saturated fatty acids. Our results are, in general, in harmony with such findings.

To confirm our results with *Staphylococcus A* other strains indicated below were tested.

Staphylococcus B: A strain recently isolated from the milk of a cow. This cow and the one from which *Staphylococcus A* was isolated were in the same herd. The characteristics are in general the same as those for *Staphylococcus A*.

Staphylococcus C: An organism from our collection of stock cultures, isolated from a lung abscess of a calf in 1918. It liquefied

gelatin but not blood serum, and showed the yellowish color of a *Staphylococcus aureus*.

Staphylococcus D: A non-hemolytic organism also from our collection, isolated from purulent milk in 1899. It shows a yellowish pigment characteristic of a *Staphylococcus aureus*.

The experiments previously described were repeated with the different strains. The data for the bouillon culture experiments are contained in Table V.

TABLE V.

Hemolytic Action of Different Strains of Staphylococci in Bouillon Containing Defibrinated Horse Blood with and without Fat.

	Strain.	Incubation period.	Cream.	Butter.	Olive oil.	Tributylin.	Triolein.	Triacetin.	Fat-free milk.	Control without fat.
		hrs.								
Blood bouillon plus culture..	Staphylococcus B	24	++++	+	+++	-	+++	-	-	-
		48	++++	+++	+++++	-	+++++	-	-	-
	" C	24	++	+	±	-	+	-	-	-
		48	++++	++	+	-	++	-	-	-
	" D	48	-	-	-	-	-	-	-	-
Blood bouillon only.			-	-	-	-	-	-	-	-

Final concentration of fat was approximately 0.002 cc. per cc. of bouillon.

The hemolytic action of Strains B and C was practically identical with that given for *Staphylococcus A*. Hemolysis occurred to a slight extent on the surface of agar plates in the absence of added fat, and was absent around the deep colonies or at most only a faint trace of hemolysis occurred. In the presence of cream, hemolysis was more extensive on the surface and occurred around the deep colonies. In bouillon, hemolysis occurred only in the presence of fat. With bouillon cultures killed with ether or chloroform the resulting fluid was able to effect hemolysis in the presence of cream. The fluids from cultures which had acted upon cream and had then been heated to 100°C.

were capable of hemolyzing red blood corpuscles. The active agent was destroyed by heating to 65°C.

Strain C which has been under cultivation for 3 years was not as active as the more recently isolated Strains A and B. The quantity of lipase elaborated in a given time was not so great, as evidenced by its slower rate of hemolysis in the bouillon culture tubes. In the tests on different kinds of fats it was found that hemolysis occurred with olive oil only after 65 hours.

TABLE VI.

Hemolytic Action of Staphylococcus A in Media Containing Blood Corpuscles from Various Animals with and without Cream.

		Horse.	Rabbit.		Sheep.	Cow.	Calf.
			A.	B.			
Agar plus culture	Without cream.	—	—	±	—	—	—
	With “	+	+	+	+	+	+
Living bouillon culture	Without “	—	+	+	+	+	—
	With “	+	+	+	+	+	+
Etherized bouillon culture	Without “	—		+	—		—
	With “	+		+	+		+
Heated bouillon culture (100°C.)	Without “						
	With “	—		+	—		—
Control with sterile bouillon		—		—	—	—	—

Final concentration of cream was approximately 0.002 cc. per cc. of bouillon. Results with agar are given for action around deep colonies.

Strain D was non-hemolytic and failed to produce hemolysis under any conditions.

The preceding experiments had been conducted with the corpuscles of one species of animal, the horse. Other animals were bled, such as sheep, rabbit, cow, and calf, and the experiments repeated with their corpuscles. The data are contained in Table VI.

In the presence of the blood corpuscles of sheep, rabbit, cow, and calf when the organism was plated on agar without the addition of cream, only a slight hemolysis occurred around the surface colonies

and no hemolysis around the deep colonies, except in the case of rabbit blood in which a very narrow hemolytic zone was present around the deep colonies. In those plates to which cream had been added hemolysis was more extensive around the surface colonies and took place around the deep colonies in a manner very similar to that for horse corpuscles. In plain bouillon with the corpuscles of sheep, rabbit, and cow, hemolysis occurred with the living culture of *Staphylococcus A* in the absence of added fat, while the result with calf corpuscles was like that with the horse. When the etherized culture fluid without cream was used, hemolysis did not occur with any of the blood corpuscles except in the case of those of the rabbit; in the presence of cream, hemolysis occurred in all cases and the extent of hemolysis with the rabbit corpuscles was much greater than in the absence of cream. Rabbit corpuscles were also hemolyzed by the heated culture. In view of the greater sensitiveness of the rabbit corpuscles, and the increased hemolysis in the presence of cream or added fat, it is not illogical to assume that there was sufficient fat in the bouillon to enable the staphylococcus to effect hemolysis through the hydrolysis of this fat. The difference between the blood corpuscles of various kinds may be due to the amount or character of fat contained in the corpuscles.

In the above experiment with the corpuscles of animals other than the horse it was noted that in the absence of cream slight hemolysis occurred around the surface colonies but not around the deep colonies. In the presence of cream, hemolysis occurred around the deep as well as the surface colonies. To eliminate the possible effect of oxygen of the air as a factor in the latter process agar shake cultures were made and pipetted into flattened test-tubes filled with the agar to a height of about 4 inches. Hemolysis took place in the bottom of such tubes as well as near the surface.

DISCUSSION.

From the experiments presented hemolysis in the case of the organism studied is shown to be associated with the presence of fat in the media. Cultures grown in plain horse blood bouillon or in the presence of fat-free milk are not hemolytic, while the cultures grown in the presence on cream or other fats are hemolytic.

The nature of the processes associated with the hemolytic action is brought out in the experiments dealing with culture fluids in which the organisms have been treated with ether, and by the effect of heat upon such fluids and on living cultures. Through the action of ether or chloroform, the organism itself is killed but this does not destroy the ability of the culture fluid to effect the hemolysis of the red blood corpuscles. If the living culture or an etherized culture fluid be heated neither of the resulting fluids is capable of hemolyzing the red blood corpuscles.¹ When a living culture or an etherized culture fluid of the staphylococcus is permitted to stand with cream or other fat for several hours and is then heated to 100°C. the resulting fluid is capable of producing hemolysis. We feel justified, therefore, in attributing the hemolytic effects to the direct action of fatty acids (or soaps) which have been formed from the cream or fat by an extracellular enzyme elaborated by the staphylococcus. The enzyme is not dialyzable, for hemolytic action could not be obtained with either living or killed cultures in collodion bags.

The occurrence of hemolysis due to the action of a lipase upon fat necessitates the consideration of two factors in the study of organisms, (a) the presence of a lipase and (b) the elimination of its lipolytic action before concluding that hemolysis is due to another type of hemolytic agent.

Many points brought out by our experiments have been observed separately by others but so far as we can find the hemolytic action of a staphylococcus has not been directly connected with its lipolytic activities. Several organisms were reported by Eijkman (1) in 1901 as producing a lipase and among these was given *Staphylococcus pyogenes aureus*. An association between hemolysis and lipolysis was demonstrated by Noguchi (2) in 1907 who showed that hemolysis may be a direct result of fat-splitting. He used various tissue lipases with a considerable number of fats, but the most satisfactory results were obtained with triolein, butter, and fatty mixtures which he extracted from the fat tissues and mesentery of dogs and guinea pigs. According to his report neither the fat nor the lipase alone produced hemolysis.

Von Liebermann (3) extracted a thermostable hemolytic substance from hog corpuscles which was found to be an acid or a mixture of acids. He did not identify the acids but was able to demonstrate similar hemolytic action with

¹ There was one exception to this statement and that was the corpuscles of the rabbit (see page 417).

oleic acid. Heating inactivated oleic acid-serum mixtures. This agrees with the work of Noguchi who found that when his alcohol extracts (soap) were heated alone they were not inactivated but when heated with serum they were inactivated. Von Liebermann was able to reactivate such mixtures by the careful addition of acid.

Neuberg and Reicher (4) demonstrated the lipolytic action of various plant and animal substances by measuring the increase in acidity of mixtures of an hemolysin and a fat, particularly olive oil. These authors suggest that the hemolytic effects observed by von Liebermann may have been due to the fatty acid produced by the action of lipase upon the fats of red blood corpuscles. In our experiments the hemolytic effect is apparently not directly upon the red blood corpuscles, since in our control experiments hemolysis failed to occur in the presence of lipases but in the absence of added fat.

Noguchi (5) also extracted a hemolytic agent from animal tissues with alcohol which consisted of soaps. Fatty acids or their soaps are known to produce hemolysis. The smallest amount of fatty acid necessary for complete hemolysis of 0.5 cc. of a 5 per cent suspension of red blood cells was studied by McPhedran (6) in 1913. In our experiments we made no quantitative determinations but we used about 0.3 mg. of the fat, and probably this was not all split into fatty acids. McPhedran found 0.03 mg. of oleic, linoleic, dibromostearic, or the two isomeric monobromostearic acids sufficient for complete hemolysis of 0.5 cc. of a 5 per cent suspension of red blood corpuscles while palmitic acid and dihydroxystearic acids were required to be present in quantities ten times as great as for the above acids.

The hemolytic character of the staphylococcus is described in various text-books as due to a specific hemolysin, staphylolysin, but no mention is made of a fat-splitting enzyme.

SUMMARY.

A staphylococcus was isolated from milk which is hemolytic with horse blood only in the presence of fat. Similar results were obtained with two other strains of staphylococci.

The hemolysis is the result of the action of a fatty acid (or soap) upon the red blood cells. The fatty acid is formed by the action of a lipase elaborated by the staphylococcus.

The corpuscles of different animals show slight variations in the ease with which they are hemolyzed by the staphylococcus.

Attention is called to the desirability of testing for lipases in the study of staphylococci or of hemolysis.

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EXPERIMENTAL RICKETS IN RATS.

IV. THE EFFECT OF VARYING THE INORGANIC CONSTITUENTS OF A RICKETS-PRODUCING DIET.*

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PLATES 21 TO 24.

(Received for publication, October 31, 1921.)

In the first paper of this series it was shown by Sherman and Pappenheimer (1, 2) that rickets could be produced in rats by a diet composed of patent flour, calcium lactate, sodium chloride, and ferric citrate, and that it could be prevented by the substitution of 0.4 per cent basic potassium phosphate for an equal percentage of calcium lactate in the diet. These experiments have been continued, the diet being modified in various ways, and the present paper will report upon the bone changes which were produced by varying the inorganic constituents.

In the meantime, there has appeared a series of studies on experimental rickets from the Johns Hopkins Laboratories in which Shipley, Park, McCollum, and Simmonds (3-7) give diets which lead more or less regularly to the development of rickets in rats, and in which they also emphasize the protective rôle of the phosphate ion. A more detailed comparison of their findings will be given later.

EXPERIMENTAL.

Because of the frequency with which rats kept under ordinary laboratory conditions develop what has previously been termed spontaneous

* A preliminary report covering some phases of this work was presented before the Society of Experimental Biology and Medicine (Pappenheimer, A. M., McCann, G. F., Zucker, T. F., and Hess, A. F., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 267).

rickets, it was realized from the outset that factors other than dietary ones which might influence the experiments should be controlled as rigidly as possible. Care was taken to obtain healthy stock, from which individual rats maintained for varying lengths of time on a complete diet showed normal bones upon histological examination. Whenever possible, controls were taken from the same litters as those used for the experiment.

The animals were kept in cylindrical wire cages, 8 inches high and 9 inches in diameter, in a dimly lighted, screened room, with northern exposure.

Since these experiments were begun it has been shown by Shipley, Park, Powers, McCollum, and Simmonds (8), and by Hess, Unger, and Pappenheimer (9) that exposure to direct sunlight gives complete protection in phosphorus-low diets. Since, fortunately, the light conditions in our experiments have been kept fairly uniform, and the rats at all times excluded from intense light, this factor has been without influence upon the results. It must be strongly emphasized, however, that our data, so far as their quantitative value is concerned, hold only for rats maintained under the described conditions.

Not more than three rats were kept in the same cage. The diets were mixed in bulk, care being taken to ensure a uniform distribution of the components. It was found impracticable to weigh back the uneaten food as a routine procedure, but the amount actually consumed was roughly determined in a number of instances and found to vary very little for animals of approximately the same age and weight. It is believed that no serious errors have been introduced by this omission, since almost without exception, the rats maintained on the same diets have shown, on final examination, identical or comparable findings in the bones.

The rats were usually placed on the experimental diet at the age of 4 weeks, or about 1 week after weaning. After 4 weeks on the test diet, a radiograph was taken; and when subsequent modifications of the diet were introduced, the accompanying changes were followed radiographically. Because of the known protective effect of light, the possibility that repeated exposure to x-rays might have a curative influence had to be considered. Three rats were exposed daily over a period of 3 to 9 days, the same dosage being used that was employed in

making radiograms. The rickets, which had developed on Diet 84, was not influenced, no healing being evident on radiographic examination.

A complete autopsy was performed on each rat, the teeth, ribs, femora, tibiae, eyes, and such viscera as showed gross lesions being preserved for histological study. Possible alterations in the teeth will be made the subject of a later report. The study of the long bones also has not been completed, but previous experience has shown that the lesions in the ribs in general parallel those found in other bones, and may safely be taken as indices of the nature and extent of the bone changes. Fig. 1 shows the microscopic appearance of the normal rib.

The difficulties in the histological examination of bones, as regards the precise differentiation of the calcified and uncalcified tissue, are too familiar to require emphasis. The routine adopted for these studies has been the following. Two lower ribs from each side were fixed for 24 hours in Müller-formol, decalcified for 5 to 10 days in Müller's fluid, and embedded in paraffin. Sections were stained with hematoxylin-eosin, and by von Kossa's silver nitrate method, applied as follows:

1.5 per cent silver nitrate—30 minutes or more in bright light.

Repeated washing in distilled water.

Counterstaining with hematoxylin-Van Gieson.

The calcium (phosphate) appears in these preparations as a dense black, the cartilage bluish purple, the osteoid tissue bright red.

The influence of the time element in the decalcification with Müller's fluid upon the stainable calcium was studied by removing ribs after varying periods from the decalcifying fluid, staining the sections identically, and comparing the results. It was found that within the range of 5 to 10 days no significant differences in the distribution of the stainable calcium could be detected.

Rickets-Producing Diet.

The rickets-producing diet, No. 84, which has served as a basis for this work, has the percentage composition indicated in Table I. The mineral composition is shown in Table II.

The original observation that rats maintained on this diet invariably develop rachitic lesions, has been tested in 55 additional animals, all

TABLE I.
Composition of the Diets Used.

Diet No.	Composition of diet.	Total No. of rats examined.	
		Rachitic.	Non-rachitic.
83	Patent flour.....95.0 per cent.	2	0
	Calcium lactate..... 3.0 “ “		
	Sodium chloride..... 2.0 “ “		
84	Patent flour.....95.0 “ “	68	0
	Calcium lactate..... 2.9 “ “		
	Sodium chloride..... 2.0 “ “		
	Ferric citrate..... 0.1 “ “		
85	Patent flour.....95.0 “ “	0	46*
	Calcium lactate..... 2.5 “ “		
	Sodium chloride..... 2.0 “ “		
	Potassium phosphate (basic). 0.4 “ “		
	Ferric citrate..... 0.1 “ “		

* Five of these were placed on special diet at the age of 60 days, one at 81 days; all remaining animals started about 1 week after weaning.

of which developed rickets as shown by x-rays, gross examinations, and by microscopic study of the ribs. (In two rats, no sections could be made.)

The preventive action of the potassium phosphate (Diet 85, Table I) has also been confirmed on thirty-one additional rats, giving a total of forty-six rats in which protection has been uniformly obtained. The mineral analysis of this diet is also shown in Table II.

Determination of the Rôle Played by Potassium and Phosphate Ions Respectively in the Protection Given by Basic Potassium Phosphate.

In this experiment, secondary sodium phosphate in the proportion of 0.8 per cent of the diet was substituted for the secondary potassium phosphate in Diet 85, this amount being calculated to contain an equivalent amount of phosphorus (0.072 gm.). Of the four rats (Nos. 88 to 91) on this diet, none developed rickets (Figs. 2 and 3). Two rats (Nos. 86 and 87) from the same litter, on Diet 84, developed marked rickets (Table III).

TABLE II.
*Calculated Mineral Composition of Diets 84 and 85.**

Mineral constituent.	Diet 84.		Diet 85.	
	Amount per 100 gm. of food.	Per cent.†	Amount per 100 gm. of food.	Per cent.†
	gm.		gm.	
K	0.110	3.8	0.290	9.4
Na	0.847	29.3	0.847	27.6
Ca	0.550	19.1	0.477	15.5
Mg	0.017	0.6	0.017	0.5
P	0.087	3.0	0.159	5.2
Cl	1.280	44.4	1.280	41.7
Total	2.891	100.2	3.070	99.9

* Data for white flour taken from Sherman, H. C., *Chemistry of food and nutrition*, New York, 2nd edition, 1918.

† On the basis that $K + Na + Ca + Mg + P + Cl = 100$ per cent.

Determinations of phosphorus and calcium (McCrudden's method (McCrudden, F. H., *J. Biol. Chem.*, 1911-12, x, 187) for calcium and titration of ammonium phosphomolybdate for phosphorus) on the diets as used gave the following figures.

	Diet 84. gm.	Diet 85. gm.
P	0.0868	0.1582
	0.0880	0.1593
Ca	0.5530	0.4805
	0.5521	0.4786

For another group of rats (Nos. 97 to 99), 0.35 per cent of potassium chloride was used, thus adding an equivalent amount of potassium (0.18 gm. per gm.) to the diet in the form of chloride instead of phosphate. The three rats on this diet developed rickets of the same degree of severity as did two control rats of the same litter (Nos. 100 and 101) on Diet 84 (Figs. 4 and 5).

The obvious conclusion from these results is that the protective effect of the basic potassium phosphate is attributable to the phosphate ion and not to the potassium.

TABLE III.

Effect of the Substitution of Sodium for Potassium, and of Chloride for Phosphate, in Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet, days	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
		No. 85A.		gm.	gm.	gm.					
88	14	Flour.....95.0 per cent.	21	31	40	40				None.*	None.
		Calcium lactate. ... 2.5 "									
89	14	Sodium chloride. ... 1.6 "	37	30	46	46	35th	None.		Slight keratitis* (left).	"
		Ferric citrate. 0.1 "									
		Sodium phosphate (basic)..... 0.8 "									
90	14		37	38	47	44	25th	"			
							35th	"		Moderate. Keratitis.*	"
91	14		37	30	47	42	25th	"		None.	Abscess in sub-inguinal gland.
							35th	"			
97	16	No. 85B.	36	38	43	40	25th	Moderate.	Moderate.*	Slight keratitis.*	None.
		Flour.....95.0 per cent.									
		Calcium lactate. ... 2.5 "									
		Sodium chloride. ... 2.05 "									
		Ferric citrate. 0.1 "									
		Potassium chloride. . 0.35 "									
98	16		21	38	46	46			Slight.*	None.	"
99	16		36	32	44	40	25th	Moderate.	Marked.*	Marked keratitis.*	"

Controls from the same litters.											
		No. 84.	27	32	40	40	24th	Marked.	Moderate.*	Marked keratitis.*	None.
86	14	Flour.....95.0 per cent.									
		Calcium lactate. ... 2.9 "									
		Sodium chloride. ... 2.0 "									
		Ferric citrate. 0.1 "									
87	14		44	33	43	40	38th	"	Marked.*	Suppuration.	Suppurative rhinitis; atelectasis of lungs.
100	16		20	34	37	32			Early.*	Slight keratitis.	None.
101	16		34	30	35	35			Slight.	Conjunctivitis.	?

* Confirmed by microscopic examination.

CORRECTIONS.

On page 429, Vol. xxxv, No. 4, under Diet 85, for 72 mg. of *phosphate*
read 72 mg. of *phosphorus*.

113	16	No. 85P. Flour..... 95.0 per cent Calcium lactate. 2.85 “ “ Sodium chloride..... 2.00 “ “ Ferric citrate. 0.10 “ “ Potassium phosphate (basic; equal to 10 mg. per 100 gm. of diet)..... 0.05 “ “	34	46	54	53	20th	Slight.	Marked.* Slight.*	Marked conjunc- tivitis. None.	None. “
118	19		27	52	58	56	20th	“			
Controls from the same litters.											
110	18	No. 84. (No phosphate added.)	27	35	40	35	21st	Slight.	Moderate.*	None.	None.
115	19		27	36	41	41	27th	Moderate.	Cod liver oil.		
111	18	No. 85. (72 mg. of phosphate per 100 gm. of diet added.)	37	46	63	63	21st	None.	Transferred to Diet 84.		
116	19		27	39	49	49	26th 20th	“ “	None.*	None.	None.

* Confirmed by microscopic examination.

TABLE V.
Effect of the Addition of Salt Mixture Complete except for Phosphorus.

Rat No.	Litter No.	Diet.	Length of time on diet. days	Weight.			Radiograph.		Pathological examination.		
				Initial. gm.	Maximum. gm.	Final. gm.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
168	29	No. 84 with Osborne and Mendel salts (without H ₃ PO ₄).	26	31	31	27	26th	Marked.	Marked.*	None.	None.
169	29	Flour.....95.0 per cent Osborne and Mendel salt mixture..... 5.0 “	26	31	31	26	26th	“	†		
227	36		28	40	48	48			Marked.*	Slight corneal opacity.	None.
228	36		37	41	46	46	35th	Marked.	Moderate.*	Slight corneal opacity.	“
229	36		37	37	43	43	35th	“	“ *	None.	“

*Confirmed by microscopic examination.

†See Pappenheimer, A. M., McCann, G. F., and Zucker, T. F., *J. Exp. Med.* 1922, xxxv, 461, Table VI.

Determination of the Amount of Phosphate Necessary for Protection.

Eleven rats (Nos. 110 to 120), including four controls, were placed on diets in which graded amounts of basic potassium phosphate had been substituted for equivalent amounts of calcium lactate in Diet 84. Additions of 10, 25, and 50 mg. of phosphorus per 100 gm. of diet in the form of K_2HPO_4 were made to the standard basic diet in this way, giving totals of 97, 112, and 137 mg. of phosphorus per 100 gm. in these diets. As shown in Table IV, of those receiving 50 mg. of added phosphorus (Rats 112 and 117), one (No. 112) showed no evidence of rickets. The other (No. 117) presents an appearance which is interpreted as slight or early rickets (Fig. 6).

The zone of proliferative cartilage is widened to six to eight cells (normally four to five). Alignment is slightly irregular and calcification of the matrix is defective in the proximal portions of the cartilage. The primary spongiosa is delicate and the trabeculae are surrounded by narrow but distinct osteoid borders about which the osteoblasts are very conspicuous. Along the shaft, endosteal bone formation is active. Around the perforating vessels, there is a distinct osteoid border which is wider than that found in normal ribs.

The rats receiving only 10 and 25 mg. of added phosphorus per 100 gm. of diet show definite rachitic lesions (Fig. 7). The proliferating cartilage is extremely wide and irregular, but the matrix, in the distal portions, especially, is not wholly free from calcium. The trabeculae of the spongiosa show an osteoid border greatly in excess of the normal, but the distinction between the fully calcified bone and the osteoid tissue is in many cases less sharp than in rats which have been on Diet 84. It would seem, therefore, that the small amount of additional phosphate favors the deposition of calcium to a limited extent, and that with the addition of 50 mg. per 100 gm. the balance is so delicate that individual differences come into play.

Effect of the Modification of Inorganic Constituents Other than Calcium and Phosphorus.

Rats 168, 169, 227, 228, and 229 (Table V) were given a diet composed of flour and 5 per cent of salt mixture made similarly to that described by Osborne and Mendel (10) except that no phosphoric acid was included (Fig. 8). These rats were kept on the diet for 26, 26, 28, 37, and 37 days and showed marked rickets.

TABLE VI.
Effect of the Reduction of Sodium Chloride in Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
		No. 84L.	days	gm.	gm.	gm.					
268	42	Flour.....96.5 per cent.	35	33	42	42	30th				
269	42	Calcium lactate. 2.9 “	35	32	40	38	30th	Marked.	Marked.*	None.*	None.
		Sodium chloride. 0.5 “							“ *	Very slight.	“
270	42	Ferric citrate. 0.1 “	35	38	48	47	30th	“	“ *	None.	“

This salt mixture as given contains an amount of calcium which is approximately that used in Diet 84, and it also contains all the other inorganic elements considered necessary for adequate growth, with the single exception of phosphorus. The development of rickets in the rats would seem to indicate that these other elements have no protective action.

The basic rickets-producing diet, No. 84, contains 2 per cent of NaCl, an amount probably considerably in excess of the requirement. It was conceivable that this excess might in some way be a contributory factor in the production of the rachitic lesions, and to settle this point, three rats (Nos. 268 to 270) were given Diet 84L, in which the NaCl was reduced from 2 per cent to 0.5 per cent. After 30 days all three rats had developed typical rickets, as revealed by gross and microscopic examination. A reduction of the NaCl in Diet 84 to 0.5 per cent, therefore, did not prevent the development of active rickets (Table VI).

Effect of Variations in the Proportion of Calcium and Phosphorus in Diets 84 and 85.

Although it had become evident from the preceding work that the presence or absence of rachitic lesions could be determined by varying the phosphate ion in the diet, when the calcium was present in excess, and that the other inorganic constituents of the food were without apparent influence, it seemed of importance to ascertain what modifications in the structure of the bones might be brought about by varying the relative proportion of calcium and phosphate in the diet.

The literature, it is true, contains many references to the structural changes produced in the bones by a calcium-deficient diet, but in general, the phosphate content of the diet in relation to the calcium has not been considered. Furthermore, aside from the recent papers of McCollum and his associates, no data referring to the effects of deficiency of one or both of these inorganic constituents upon the bone structure of rats were available.

A summary of our experiments is given in Table VII. In the first series (Rats 233 to 235), the calcium lactate and basic potassium phosphate of Diet 85 were reduced, the former to 1.4 per cent (209 mg. per 100 gm. of diet), the latter to 0.26 per cent (49 mg. of added P, or a total of 136 mg. per 100 gm.).

TABLE VII.
Effect of Variations in the Proportion of Calcium and Phosphorus in Diets 84 and 85.

Rat No.	Litter No.	Diet.	Length of time on diet.			Weight.			Radiograph.		Pathological examination.		
			days	Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Rickets.	Ophthalmia.	Other lesions.
		No. 85 + 209 mg. of Ca.		gm.	gm.	gm.							
233	38	Flour. 96.5 per cent.	41	31	54	54	28th	Slight.			Slight, healing.*	None.*	None.
234	38	Sodium chloride. 2.0 "	41	35	68	67	28th	Moderate.			Healing.*	"	"
235	38	Calcium lactate. 1.14 "											
		Potassium phosphate (basic). 0.26 "	30	31	46	46	28th	"			Slight, healing.*	" *	Hymenolepis murina.
		Ferric citrate. 0.1 "											
239	38	No. 85 + 149 mg. of Ca.											
		Flour. 96.97 per cent.	30	30	53	53	28th	Moderate.			None* (increase in width of paratary cartilage).	None.	None.
		Sodium chloride. 2.0 "											
		Calcium lactate. 0.8 "											
		Potassium phosphate (basic). 0.13 "											
240	38	Ferric citrate. 0.1 "	42	28	50	50	28th	Slight.			None* (increase in width of paratary cartilage).	" *	Suppurative nephritis.
236	38	No. 84 + 111 mg. of Ca.											
		Flour. 97.3 per cent.	30	34	52	52	27th	Marked.			Marked.*	None.*	None.
237	38	Sodium chloride. 2.0 "	42	29	58	55	27th	"			" *	"	"
238	38	Calcium lactate. 0.6 "											
		Ferric citrate. 0.1 "	42	27	54	54	27th	"			" *	"	"

* Confirmed by microscopic examination.

At the end of 4 weeks, the radiographs showed that all three rats had developed an active rickets. Rat 235, killed at this time, had a mild healing rickets, with calcium deposition in the cartilage and in the osteoid surrounding the trabeculae of the spongiosa. Rat 234, allowed to live for 41 days, showed microscopically definite healing, the cartilage and the greater portion of the osteoid being completely calcified. Rat 233, killed on the 42nd day, likewise showed healing rickets, the calcification of the cartilage and osteoid being somewhat less advanced than in the ribs of Rat 234.

In this experiment, the total P of the diet (136 mg.) was practically at the border-line content which previous experiment (Table IV, Rats 112 and 117) had shown to confer protection in some rats and not in others. The results, therefore, are not surprising—rickets after 4 weeks, with adequate healing after 41 days. It does not appear that the coincident reduction of the calcium has materially modified the lesions.

In two other rats of the same litter (Nos. 239 and 240) the calcium lactate of Diet 85 was reduced to 0.8 per cent (149 mg. of Ca per 100 gm.), and the basic potassium phosphate to 0.13 per cent (total P of 111 mg. per 100 gm.).

Again, both rats showed by radiograph, on the 28th day, an appearance interpreted as moderate rickets. Rat 239, killed on the 30th day, proved not to have rickets, the cartilage, spongiosa, and cortex being normally calcified. There was, however, an increase in the depth of the zone of preparatory cartilage to ten or twelve cells, which explains the defect noted in the radiograph. Rat 240, killed on the 42nd day, was found to have practically complete healing of the rachitic lesions in the ribs.

The zone of preparatory calcification, though wider than normally, averaging twelve to sixteen cells, and continued into several irregular prolongations, showed a complete calcification of the matrix. The primary spongiosa was somewhat defective, the secondary spongiosa better developed. In both, calcification was complete, there being no osteoid tissue about the trabeculae. The cortex also showed no osteoid border. The trabeculae in the metaphyseal area were separated by a very abundant, loose fibrous marrow. The perichondral osteoid was still calcium-free.

The conditions found in this rat may instructively be compared with those in Rat 120, maintained for 40 days on Diet 85O, containing an equal amount of P, but almost four times as much Ca. So far as it is permissible to draw inferences from a single experiment, it would seem that the healing of the rachitic lesions was favored by the reduction of the calcium; or otherwise stated, that the excess of calcium in the diet tended to maintain the rachitic condition.

In another series (Rats 236 to 238), no additional phosphate was given, but the calcium lactate of Diet 84 was reduced to 0.6 per cent (111 mg. of Ca per 100 gm.). The three rats, after 30, 42, and 42 days respectively, developed rachitic lesions typical histologically, and not differing from those observed on the unmodified Diet 84. The deduction from this experiment is that the Ca may be reduced to approximately one-fifth that of Diet 84, and if there is a concomitant P deficiency, the resultant lesions will be rachitic and not osteoporotic.

Effect of a Deficiency of Calcium with an Excess of Phosphate.

The material for this experiment consisted of fourteen rats, examined after periods of 29 to 36 days, upon the following diet.

Diet 85C.

Patent flour	95.0 per cent.
Sodium chloride	2.0 " "
Potassium phosphate (basic)	2.9 " "
Ferric citrate	0.1 " "

This diet led to alterations in the structure of the ribs resembling the usual rachitic changes on Diet 84, but with certain striking and interesting differences (Fig. 9). The zone of preparatory calcification of the cartilage in many of the rats was but slightly greater than in normal ribs, and in some cases within the normal range, not exceeding five or six cells. In only two rats of this series (Nos. 243 and 244) did the increased depth approximate that seen on Diet 84.

The calcium deposition in the matrix of the cartilage was also decidedly more abundant than in rats on Diet 84. In some, the calcium was found throughout the zone of preparatory calcification, in others it was limited to the matrix between the distal cells. In general, there was a much more adequate preparatory calcification of the cartilage

than was obtained in rats upon a diet rich in calcium, but deficient in the phosphate ion.

The alterations in the bony portions of the ribs, on the other hand, did not differ materially from those occurring in rats on Diet 84. There was an excessive amount of calcium-free osteoid tissue produced, not only about the trabeculae in the subchondral region, but along the shaft as well. The osteoblasts were numerous and conspicuous.

The following tentative explanation is presented. It is probable that the matrix of the cartilage in the zone of provisional calcification has a greater avidity for both the calcium and phosphate ion than the osteoid tissue of the bone itself, since it has been observed that in the process of healing under the influence of cod liver oil, or following the addition of phosphate to Diet 84, the deposition of calcium in the cartilage precedes that in the osteoid tissue. This partial calcification of the cartilage suffices to guide the blood vessels towards a more orderly destruction of the cartilage columns, and to prevent the unrestricted and highly irregular growth of the cartilage towards the diaphysis, which takes place when, as in Diet 84, the cartilage matrix remains permanently free of calcium.

To sum up this experiment, then, we may conclude that a diet containing an abundance of phosphate, but deficient in calcium, leads to changes in the bones which resemble those of rickets, but differ from them in the fact that the endochondral changes are relatively slight or absent.

The typical findings on this diet are illustrated by the following protocol.

Rat 245.—36 days on Diet 85C. Initial weight 35 gm. Final weight 38 gm. The radiograph, taken on the 36th day, showed a narrow defect in the upper epiphyses of the tibiae.

Macroscopic Examination.—Nutrition poor. *Eyes.*—Normal. *Viscera.*—Normal. *Ribs.*—Slight chondrocostal swelling, without angulation. The epiphyseal line is quite sharp. There are several fusiform callus beads. The bones are soft. *Tibia.*—The upper extremity is moderately swollen, and there is an increased amount of soft spongiosa in the subchondral region.

Microscopic Examination.—*Rib.*—The preparatory cartilage is very little widened, not exceeding eight cells (normally two to six). The columnar arrangement of the cells is maintained. Calcium deposition is limited to the matrix between the distal two or three cells. The subchondral zone is occupied by broad

masses of osteoid, the marrow in this region being reduced to a few vascular clefts. The osteoblasts are conspicuous and, in many places, surrounded by osteoid. The centers of the osteoid trabeculæ are composed of calcified bone and cartilage. The junction between calcified bone and osteoid is fairly sharp, although there is often a granular purple haze surrounding the calcified bone which suggests that the adjacent osteoid is not wholly free from calcium. The cortex is much thinned in places and even completely interrupted. It is everywhere surrounded by a wide osteoid margin. *Tibia*.—Upper epiphysis: The lesions here are very slight. The zone of preparatory calcification of the cartilage is little, if at all, increased in depth, not exceeding six cells, and the cartilage cells show a very orderly columnar alignment. There are no projections, the line being even and sharp. Calcification of the matrix is complete in the distal three or four cells. The spongiosa is quite dense. There are numerous small trabeculæ, well ossified, but with slightly increased osteoid borders. The osteoblasts are swollen and very distinct; there appears to be very active osteogenesis. Cortex further along the shaft is well calcified. There is active endosteal bone formation, with a distinct, but hardly excessive osteoid margin. The rachitic changes are insignificant as compared with those in the ribs.

Diagnosis.—Atypical rickets.

Effect of a Deficiency of Both Calcium and Phosphate.

The following diet was used for this study.

Diet Q.

Patent flour	97.9 per cent.
Sodium chloride	2.0 " "
Ferric citrate	0.1 " "

The estimated Ca content is 0.018 gm., as compared with 0.553 gm. of Ca content in Diet 84. The P content is approximately 0.088 gm. per 100 gm., or about five times that of the Ca. Eleven rats (Table VIII) were placed on this diet for periods varying from 20 to 52 days.

In our preliminary paper (1), it was stated that rats upon this, or rather a similar diet, without the addition of the ferric citrate, developed an osteoporotic condition of the bones, and not a true rickets. Further experience leads us to modify this statement somewhat. Of the nine additional rats, one only (No. 254) showed lesions which may be regarded as purely osteoporotic.

The conditions in the remaining rats differ in certain features from those of simple osteoporosis, and from rickets, as seen in rats on Diet 84. In the radiographs, the defect at the upper extremity of the tibia

TABLE VIII.
Effect of a Deficiency of Both Calcium and Phosphorus.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		Remarks.
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.		
			days	gm.	gm.	gm.					
796	?	Patent flour. 98.0 per cent. Sodium chloride. 2.0 "	52	41	44	44			Atypical.*		
799	?		52	35	39	35			" *		
252	40	Diet Q. Patent flour. 97.9 per cent.	34	39	51	?	31st	Slight defect.	Atypical.*		Ate part of rat.
254	40	Sodium chloride. 2.0 "	21	37	39	33			Simple osteoporosis.*		
255	40	Ferric citrate. 0.1 "	34	31	38	38	31st	Slight defect.	Slight, atypical.*		Ate part of Rat 254.
480	61		26	52	60	59	26th	"	Atypical.*		
481	61		32	60	71	71	26th	"	" *		
482	61		32	57	73	73	26th	"	" *		
492	62		32	27	38	38	26th	"	" *		
493	62		26	26	37	37	26th	"	" *		
494	62		32	27	44	44	26th	"	" *		
483	61	Full.	35	58	138	138	30th	None.	None. *		
484	61		35	56	134	134	30th	"	" *		
490	61	No. 84.	26	50	57	57			Typical*		

* Confirmed by microscopic examination.

was very much narrower than in the typically rachitic rats. The swelling of the chondrocostal junctions was slight or absent, and there was no angulation or other deformity. Infractures with callus beads were seen in some of the rats, and were absent in others. The bones were delicate and fragile, rather than pliable.

Histologically, the zone of preparatory calcification was somewhat increased in depth, but not to the same extreme degree as with Diet 84, averaging only eight to twelve cells, whereas in the typically rachitic rats on Diet 84 it frequently was more than 50 cells deep. Calcification of the matrix was either absent entirely or limited to the distal portion of this zone and to the short prolongations into the metaphysis. The perichondral osteoid was only moderately increased. The trabeculae of the subchondral spongiosa showed a wide osteoid margin, and there was a moderate increase in the cortical osteoid. Evidences of increased osteoclastic resorption were not found. The marrow showed no unusual features (Fig. 10).

These findings in general resemble rather closely those with Diet 85C, in which a deficiency of calcium is combined with an excess of phosphate. It should be noted that even in Diet Q, the proportion of total phosphorus to calcium is still in excess of that in $\text{Ca}_3(\text{PO}_4)_2$.

Effect of a Deficiency of Phosphate upon the Bones of Adult Rats.

Although somewhat apart from the main problem, it may be of interest to describe briefly the changes in the bones of mature rats after the cessation of epiphyseal growth, when placed upon a phosphorus-deficient diet. Three such rats (Nos. 22, 55, and 56) have been studied. Rat 22, after 182 days on a complete diet, during which period it had shown normal growth, was placed for 10 days on Diet 84. Rats 55 and 56, after 126 days on a complete diet, were placed for 42 days on Diet 84.

The histological changes in the ribs of these three rats were practically identical. The zone of preparatory calcification was very narrow (three to four cells), and the cells were separated by a densely calcified matrix. The abnormality noted was a definitely increased osteoid margin about the trabeculae of the spongiosa and along the endosteal surface of the cortex. This osteoid tissue, which was very sharply differentiated from the calcified bone, was bordered externally by distinct

osteoblasts. Without entering into the still debatable question as to whether this calcium-free osteoid is to be regarded as newly formed, or whether it results from a decalcification of the already calcified bone, we may record simply that phosphorus deficiency manifests itself in the adult rat by an increase in the amount of osteoid, and that the calcification of the cartilage remains for at least 42 days unimpaired. It is well recognized that endosteal bone formation persists, though at a more sluggish rate, throughout adult life; and the simplest explanation of the appearance described is that the newly formed bone, in the absence of an adequate supply of phosphate, remains uncalcified.

DISCUSSION.

The main facts brought out in this study may now be briefly reviewed. The basic observation has been amply confirmed that lesions of the bones identical with those previously described in rats as spontaneous rickets, may be unfailingly produced by subjecting the animals to a diet containing an adequate amount of calcium, but deficient in phosphates. When the phosphate ion is added in adequate amount (equivalent to 75 mg. of P per 100 gm. of diet), the development of rickets is invariably prevented. The border-line, where individual differences come into play, is in the neighborhood of 50 mg. of added P, and when less than this is given, rachitic changes in the bones result.

It could easily be shown, moreover, that neither the potassium in itself, nor any of the other inorganic salts which enter into a complete diet, confer any protection, if there is at the same time a deficiency of the phosphate ion.

Equally definite and striking, though possibly bearing less upon the problem of human rickets, has been the observation that lesions resembling rickets may also follow the administration of a diet deficient in calcium, but containing an adequate or excessive amount of phosphate. The lesions observed have differed from those resulting from a deficiency of phosphate in the presence of an adequate or excessive amount of calcium, in that the endochondral changes are less marked. The lesions which resulted from a diet highly deficient in both calcium and phosphorus differed from the more typical rickets in degree rather than in kind. With one exception, there developed a moderate increase in the depth of the zone of preparatory calcification, with defective

deposition of calcium, and an excess of osteoid in the spongiosa and cortex. There was no clear anatomical evidence of an increased resorption of calcium. The effects of altering the proportion of calcium and phosphate in the diet are, perhaps, less easily interpreted. It seems certain that a marked excess of Ca, such as enters into the composition of Diet 84, is not essential to the production of rickets. In our experiment, although the Ca was reduced to one-fifth of the original amount, marked rachitic lesions still developed. Even with the reduction of the calcium to 0.018 gm. per 100 gm. of diet the lesions, as has been stated, were those of an atypical rickets rather than an osteoporosis. This is what one might expect, in view of the fact that, within wide limits, the blood Ca is independent of the Ca intake.

In reviewing the literature for comparable experiments, we should mention first the studies of the Johns Hopkins investigators. Their fundamental observation that rachitic changes may be regularly induced on a diet deficient in phosphate ion, and that the introduction of the phosphate ion into the diet prevents the rickets, is in accord with our own work. A quantitative comparison from the point of view of the phosphorus content of the diet is somewhat difficult, because it is not clearly stated in their papers which diets led to rickets and which to osteoporosis. In their third paper (5) two diets are given, one (Lot 2667) containing 0.2165 gm. of phosphorus per 100 gm. of diet, the other (Lot 2806), 0.1580 gm. On these two diets the rats showed lesions ranging from simple osteoporosis to florid rickets, but it is not stated whether the more typically rachitic rats were those on the diet containing the lower amount of phosphorus. In our experience Diet 2806 would have been on the border-line; Diet 2667 would lead only to osteoporotic changes. A third diet (Lots 3120 and 2815) contained 0.5383 gm. of phosphorus, and on this the lesions were typically osteoporotic.

In Paper V (6) another rickets-producing diet is given (Ration 3127) in which the phosphorus content is stated to be 0.209 gm. per 100 gm. of diet. This is decidedly above the amount which we have found to give protection. The discrepancy may be due to the form in which the phosphorus was given, to differences in the amount of roughage, or to factors as yet undetermined.¹

¹ Since this paper was submitted, Eddy, Muller, and Heft (Eddy, W. H., Muller, H. R., and Heft, H. L., *J. Biol. Chem.*, 1922, L, p. xix) have presented experiments indicating that phytin phosphorus added to Diet 84 in double the amount supplied by the inorganic phosphate of Diet 85 fails to prevent rickets. In the diets used by McCollum and his coworkers, a large proportion of the total phosphorus must be assumed to be present in the form of phytin phosphorus. This may account for the discrepancy as regards the minimal amount of phosphorus required to protect against rickets.

A recent paper by Korenchevsky (11) also affords an interesting comparison. Negative results were obtained in phosphate deficiency experiments, but these may be explained, as the author himself points out, by the relatively high phosphorus content (0.220 gm.) of his basic diet, which is well above our protective level of 0.160 gm. per 100 gm.

While our quantitative figures obviously have no claim to finality, since the actual phosphorus intake will vary with the character of the diet and the general condition of the individual rat, we believe from our experience that it is possible to approximate very closely the phosphorus requirement for normal calcification, when the experimental conditions as regards light, age, rate of growth, and type of diet are standardized.

The question has been raised as to whether it is justifiable to apply the term experimental rickets to a condition which seems as yet unrelated etiologically to the human disease. We believe so, and for several reasons. The term rickets is habitually used in an anatomical sense, as referring to certain well characterized changes in the bones. That such alterations may be produced in various ways, and that factors other than dietary enter into the causation of human rickets may be freely admitted. But these considerations, it would seem, should not affect the propriety of terming the experimentally produced lesions rachitic, provided they conform accurately to the lesions which characterize the human disease.

Furthermore, the application of the facts observed in these experiments may not be so alien to the etiology of human rickets as would seem on first thought. A gross deficiency of calcium or phosphorus does not, it is true, occur under ordinary conditions; and rickets commonly develops in infants in spite of what appears to be an adequate supply of these inorganic substances in the food. It cannot be said, however, that we possess an accurate knowledge of the phosphorus requirement of the growing child, nor can it be said dogmatically that the amount contained in milk, for example, is in all cases sufficient without the adjuvant action of sunlight. The recent observations of Howland and Kramer (12), and confirmatory studies which will be reported in full elsewhere, indicate that there is, in active human rickets, and in the experimental rickets of rats produced by phosphorus-low diets, a striking deficiency in the inorganic phosphates of the blood. How this

is brought about remains to be investigated, but it has not been disproved that the proximate cause of rickets, in infants as well as in rats, lies in an inadequate supply of phosphate to the bones.

CONCLUSIONS.

1. Rachitic bone lesions may be produced in rats by a diet containing an excess of calcium, but deficient in phosphates.

2. Similar lesions follow a diet deficient in calcium, but containing an excess of phosphates. The endochondral lesions, however, are less pronounced.

3. A diet deficient in both calcium and phosphate induces atypical rickets.

4. Inorganic salts other than calcium or phosphate seem to be without influence upon the development or prevention of rachitic lesions.

5. The minimal amount of phosphate (calculated as P) required for normal calcification in young growing rats (30 to 50 gm.) on the diets used in these experiments is approximately 160 mg. per 100 gm. of diet. At 135 mg. individual variations come into play; at 110 mg., or less, rickets invariably follows.

6. An excess of calcium is not necessary to the production of the rachitic lesions. A reduction of the calcium lactate of Diet 84 to 0.6 per cent (111 mg. of Ca per 100 gm. of diet) still results in the development of typical rickets.

7. Adult rats react to a deficiency of phosphate in the diet by the production of calcium-free osteoid in abnormal amount about the spongiosa and cortex. There is not produced any alteration of the epiphyseal cartilage, as in growing animals.

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EXPLANATION OF PLATES.

PLATE 21.

FIG. 1. Rat 82. 52 days on Eiweissmilch. Normal growth. Normal rib.

FIG. 2. Rat 90. Diet 84 plus 0.8 per cent secondary *sodium* phosphate. Radiograph showing normal bones on 35th day.

FIG. 3. Rat 89. 35 days on Diet 84 plus 0.8 per cent secondary *sodium* phosphate. Rib: The zone of proliferating cartilage is about four cells deep, the matrix between the columns is calcified. The primary spongiosa is very defective, and epiphyseal growth in abeyance. Secondary spongiosa also scant. Cortex narrow; an endosteal osteoid margin bordered by osteoblasts is visible in a few places, but is not wider than in normal bones of young rats. No rickets. 11 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 22.

FIG. 4. Rat 97. 25 days on Diet 84 plus 0.35 per cent *potassium chloride*. Radiograph showing marked rickets.

FIG. 5. Rat 99. 36 days on Diet 84 plus 0.35 per cent *potassium chloride*. Rib: Zone of proliferating cartilage greatly widened with deep prolongations. Matrix uncalcified. Spongiosa represented by dense mass of Ca-free osteoid, with narrow Haversian spaces between the broad and convoluted trabeculae. A mass of osteoid fills in the angular depression on the external surface of the chondrocostal junction. Wide osteoid margin about cortex. Perichondrium and periosteum thickened. Marked rickets. 11 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 23.

FIG. 6. Rat 117. 34 days on Diet 85N (Diet 84 with the addition of basic potassium phosphate, equivalent to 50 mg. of P per 100 gm. of diet). Slight or early rachitic lesions (see text). 6 days decalcification in Müller's fluid. Silver nitrate-Van Gieson stain.

FIG. 7. Rat 119. 34 days on Diet 85O (Diet 84 with the addition of basic potassium phosphate equivalent to 25 mg. of P per 100 gm. of diet). More marked rachitic lesions than on Diet 85N. 6 days decalcification in Müller's fluid. Silver nitrate-Van Gieson stain.

PLATE 24.

FIG. 8. Rat 168. 26 days on Diet 84 plus Osborne-Mendel salt mixture without phosphoric acid. Radiograph showing marked rickets.

FIG. 9. Rat 392. 29 days on Diet 85C. The zone of preparatory calcification averages ten to twelve cells, not regularly aligned in columns. There are no deep projections into the metaphysis. Calcium is laid down between the distal three or four cells, but is defective in the central portion. The spongiosa is composed of broad trabeculae of osteoid, with a narrow central core of calcified cartilage or bone. The perichondral osteoid is calcium-free and slightly broader than normally. The calcified cortex is thin, and covered on both sides by a broad margin of osteoid. There is irregular fibrous periosteal thickening. The margin between calcified bone and osteoid is everywhere sharp. The lesion differs from early rickets only in the relatively slight alterations of the cartilage.

FIG. 10. Rat 493. 26 days on Diet Q. Zone of preparatory calcification eight to ten cells in depth and entirely calcium-free. Much subchondral and cortical osteoid. The apparently calcified bone stains faintly with hematoxylin, and gives no reaction with silver nitrate. 5 days decalcification in Müller's fluid.

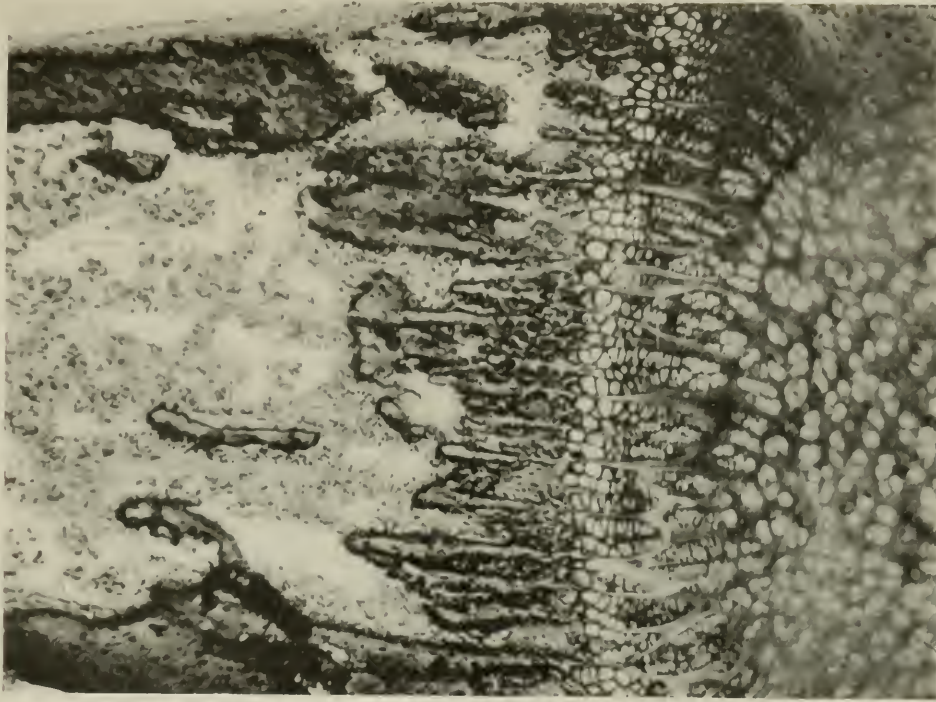


FIG. 1.

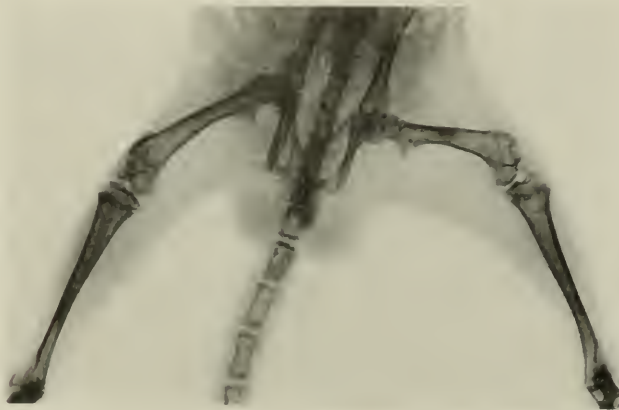


FIG. 2.

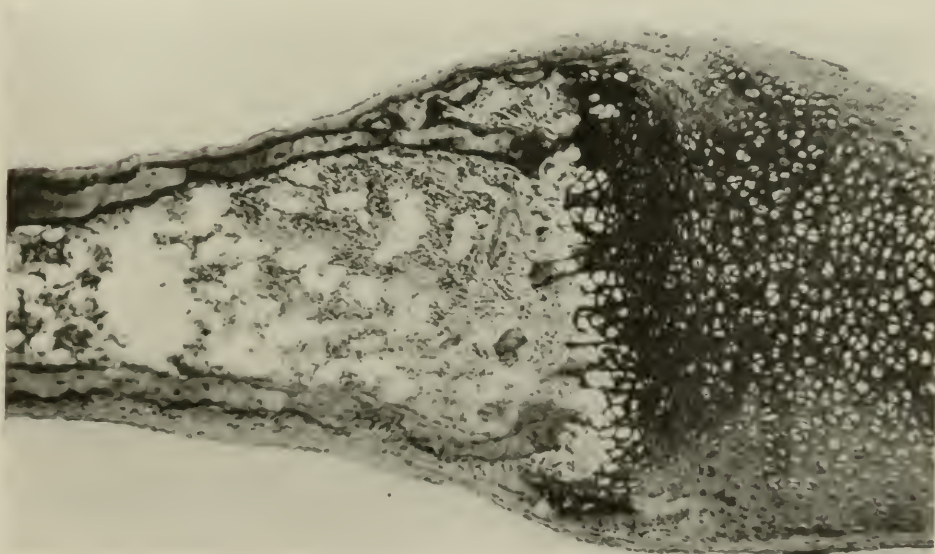


FIG. 3.

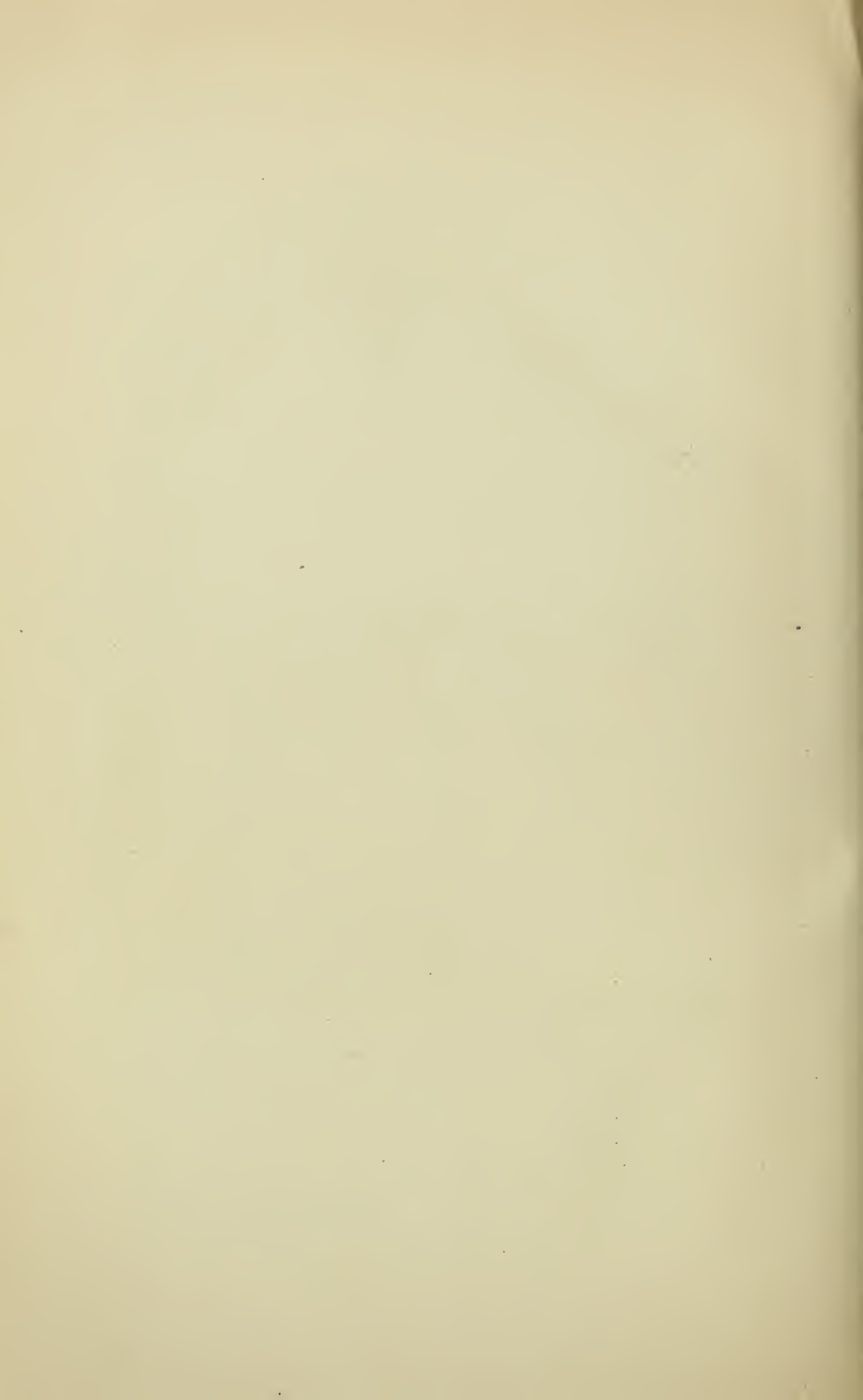




FIG. 4.

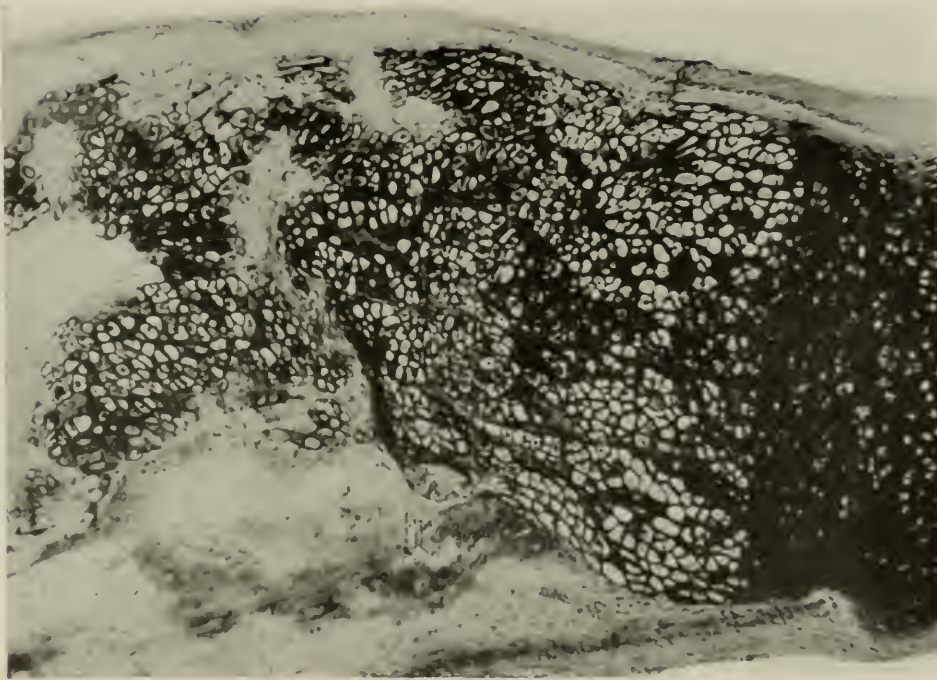


FIG. 5.

(Pappenheimer, McCann, and Zucker: Experimental rickets. IV.)





FIG. 6.

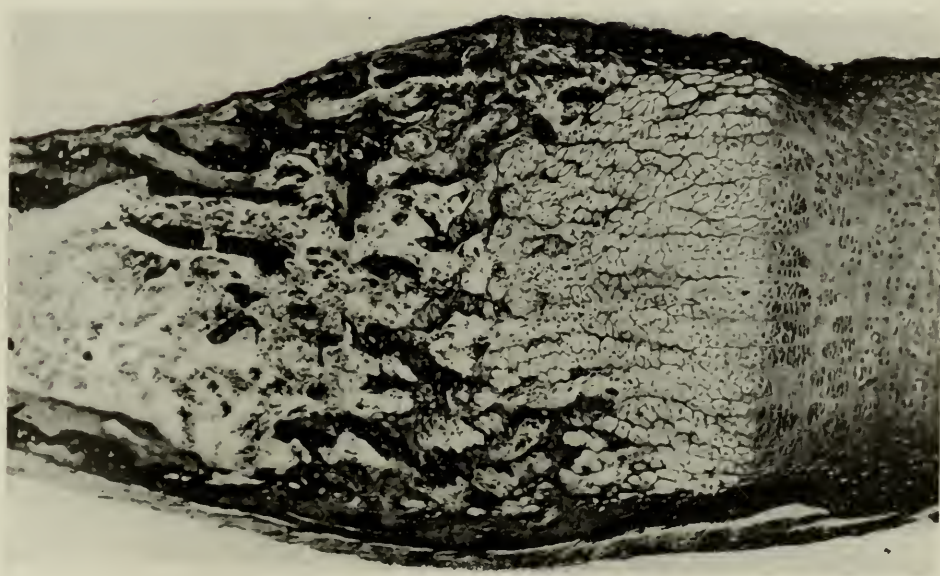


FIG. 7.

(Pappenheimer, McCann, and Zucker: Experimental rickets. IV.)

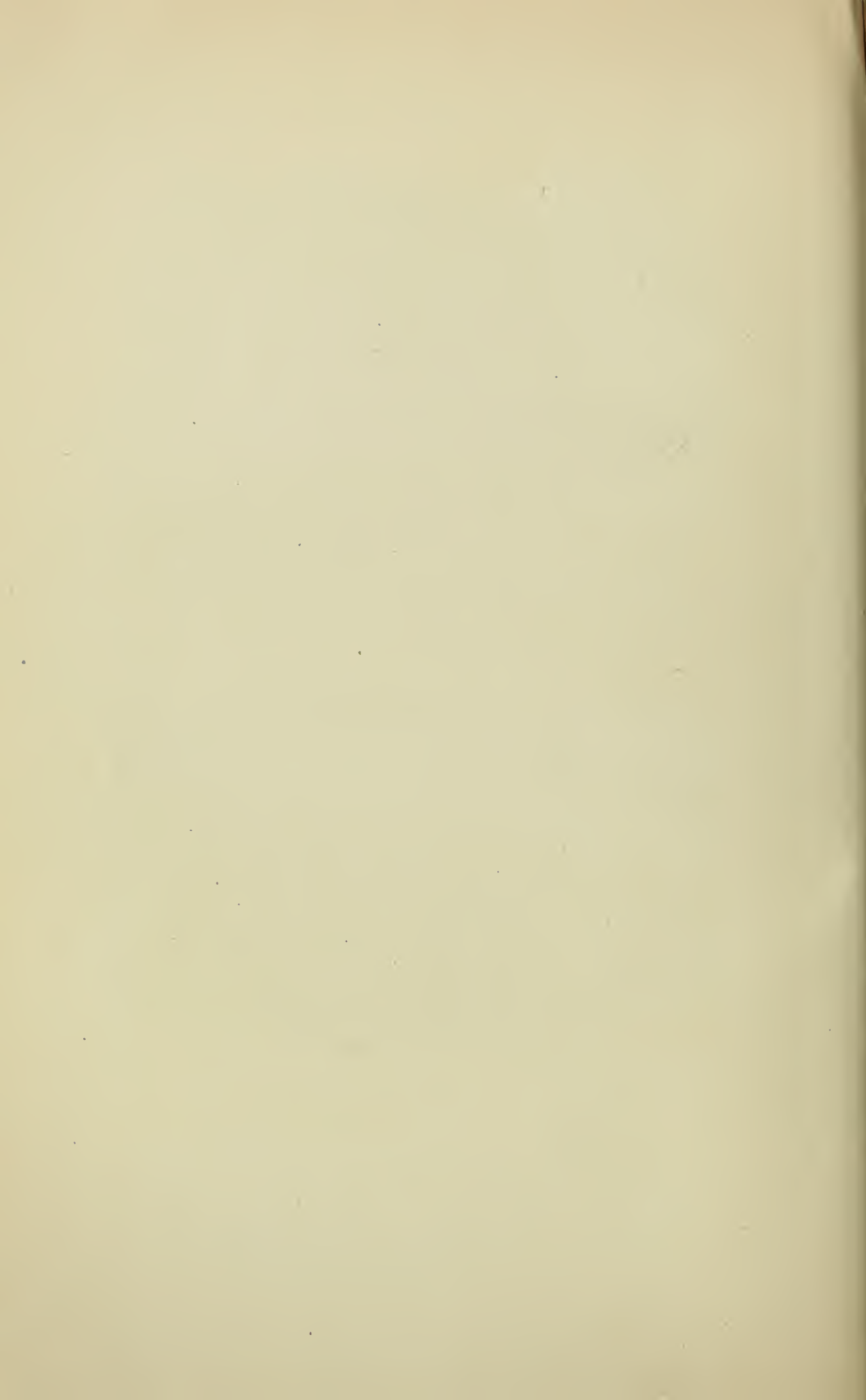




FIG. 8.

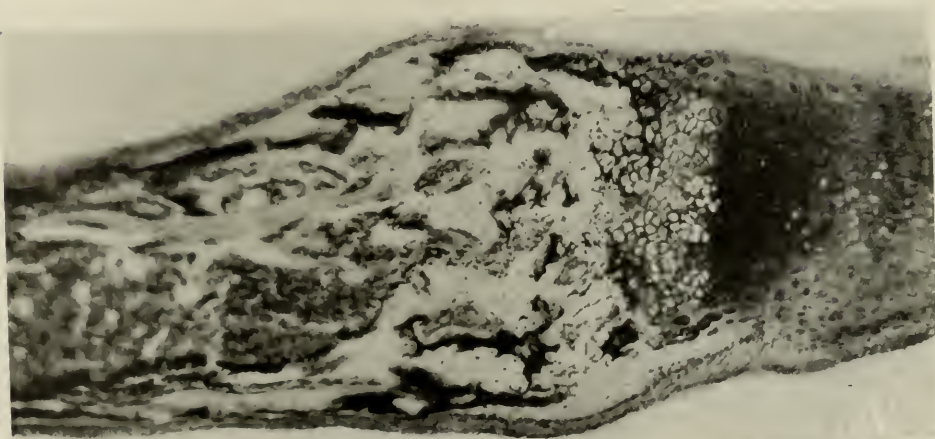


FIG. 9.

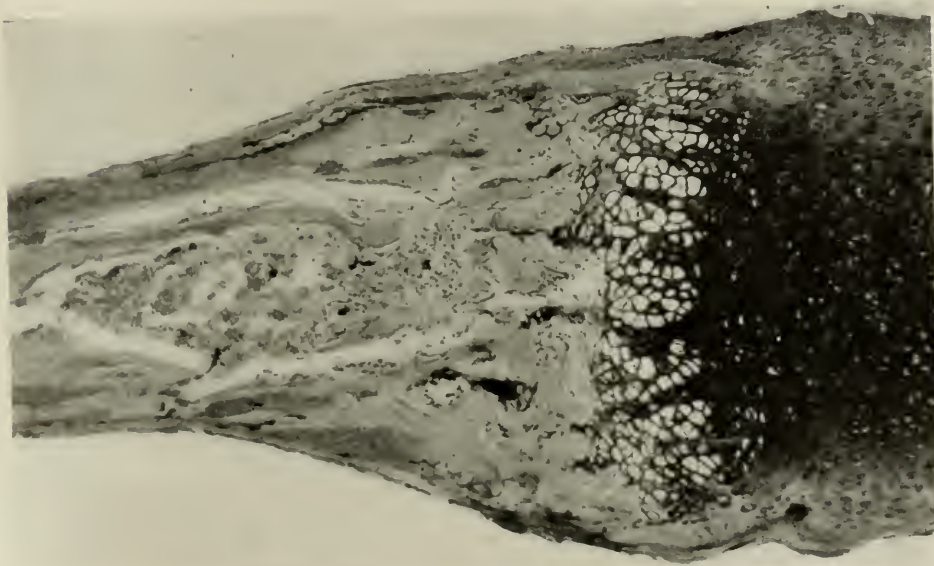
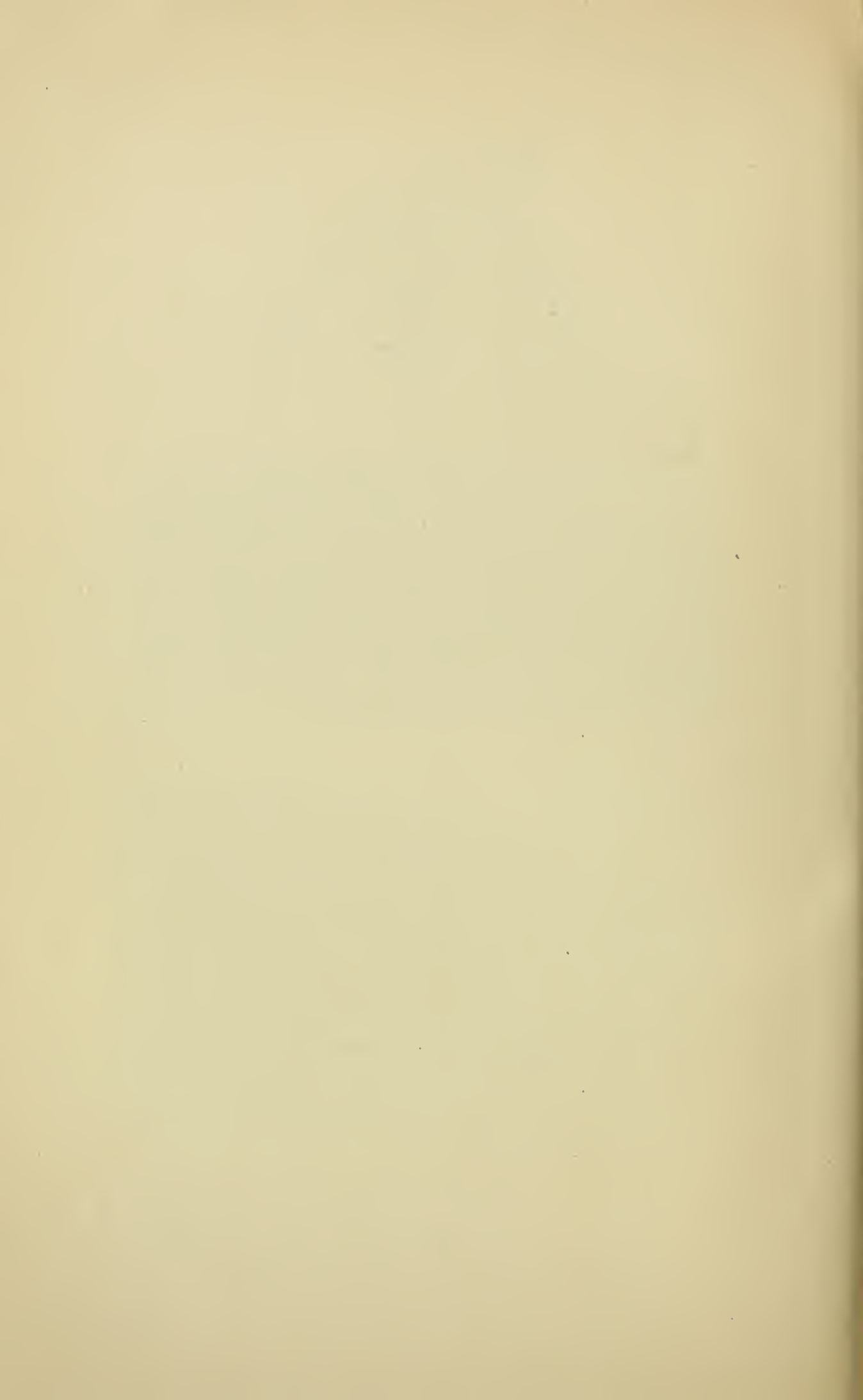


FIG. 10.

(Pappenheimer, McCann, and Zucker: Experimental rickets. IV.)



EXPERIMENTAL RICKETS IN RATS.

V. THE EFFECT OF VARYING THE ORGANIC CONSTITUENTS OF A RICKETS-PRODUCING DIET.*

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PLATES 25 TO 29.

(Received for publication, October 31, 1921.)

In a preceding article (1) it was shown that rachitic lesions may be regularly induced in young growing rats by a deficiency of PO_4 in the diet, provided the Ca ion is present in sufficient quantity; and that a deficiency of both Ca and PO_4 , or of Ca alone leads to bone changes which differ in certain respects from those of typical rickets.

The present study is concerned with the bone changes which result from modifying the organic constituents of the rickets-producing diet. We have sought, in particular, an answer to the following questions.

1. Is phosphorus, ingested in organic combination, of equal value, as compared with inorganic phosphorus, for the purposes of bone formation?

2. Do the water-soluble and fat-soluble vitamins exert any protective or curative effect, apart from the phosphorus content of the substances in which they occur? It has already been shown that the rachitic lesions which follow a diet lacking in phosphates may be prevented by merely remedying this deficiency (2), and that rats maintained throughout their growth period upon a diet deficient in fat-soluble vitamins, but adequate as regards salt content, fail to become rachitic (3). It was still conceivable that the addition of the fat-soluble factor to the rachitic diet might exercise some protec-

* A preliminary report of this work was presented before the Society of Experimental Biology and Medicine (1).

tive influence. A possible influence of the water-soluble factor also remained to be investigated.

3. Is it possible to make the rickets-producing Diet 84 more nearly adequate for proper nutrition and growth, without impairing its rickets-producing effect? Rachitic infants are often well nourished, whereas Diet 84, as has been pointed out, is not adequate for the normal growth and nutrition of rats. It was an obvious problem to attempt to bridge this discrepancy in amplifying Diet 84 by the addition of the lacking food constituents.

The experimental procedure has been fully detailed in the preceding paper, and need not be again described.

Effect of the Addition of Casein to Diet 84.

In order to study the effect of phosphorus in the form of phosphoprotein, 10 per cent of casein was substituted for the same percentage of flour in Diet 84. The casein used was the ordinary commercial product which had been extracted with cold alcohol by slow percolation until the washings were colorless, and then extracted for 48 hours with two changes of ether. Analysis of this casein showed a P content of 0.840 per cent. The diet contained approximately the same amount of phosphorus (162 mg. per 100 gm.) as Diet 85.

The results of this experiment, as shown in Table I, require further comment. Rat 95, which showed moderate rickets on x-ray examination, was killed on the 22nd day (Fig. 1). Gross examination showed a slight beading and haziness of the chondrocostal junctions. Microscopically, there were found rachitic lesions of moderate intensity (Fig. 2).

Rat 95.—Rib: The zone of proliferative cartilage is very broad, averaging twenty-five to thirty cells in depth. From this, single or double rows of cartilage cells extend into the metaphysis, and between these the matrix is calcified. No demonstrable calcium, however, is present in the proximal or basal portion of the zone of preparatory calcification. There is a very dense spongiosa composed for the most part of uncalcified osteoid tissue. A few of the trabeculæ show an inner ossified tissue enveloping the calcified cartilaginous matrix. The osteoid tissue is more than normally abundant along the cortex, especially about the perforating vessels. The marrow space of the shaft is broad, the marrow cellular. The lesion may be interpreted as early healing of a moderate rickets.

The other two rats of this series (Nos. 104 and 105) showed in radiographs taken on the 22nd day (Fig. 3) slight but definite changes which were interpreted as rachitic. Radiographs taken on the 36th day, however, showed apparently complete healing (Fig. 4). No rachitic deformities were found at autopsy, nor did microscopic study of the ribs show evidence of the previous lesion (Fig. 5).

These experiments strongly suggested that the casein phosphorus conferred a less complete protection than would be given by an equivalent amount of phosphorus in the form of basic potassium phosphate. The following experiments confirm this. Three rats (Nos. 292 to 294) were placed on Diet 84 with 5 per cent purified casein substituted for an equal percentage of flour. After 28 days all three rats showed, by radiograph, marked rickets; and one rat sacrificed at this time showed severe deformities and characteristic microscopic lesions without healing. Three other rats (Nos. 295 to 297) were given 15 per cent of casein, equivalent to a total P content of 200 mg. per 100 gm. of diet—an amount considerably in excess of that required to afford complete protection when administered as inorganic phosphate. Of these, all showed by x-ray examination on the 28th day moderate rickets. In one rat (No. 296), however, healing was apparently in progress as judged by the presence of a definite transverse shadow in the head of the tibia. Another rat (No. 297) killed on this day and examined microscopically proved to have moderate rachitic lesions with partial healing. The third rat of this group (No. 295) showed no healing radiographically on the 37th day. It was found dead in the cage and too decomposed for histological study.

The only conclusion that can be drawn from this series is that the protection afforded by casein during the period of most active growth is not wholly equivalent to that given by the same amount of phosphorus in the form of basic potassium phosphate. We have as yet no explanation for the discrepancies shown by individual rats. The difference cannot be correlated merely with the differences in the rate of growth.

Effect of the Addition of Lecithin to Diet 84.

Lecithin was added to the diet with the view of ascertaining whether phosphorus in the form of a phospholipin exerts an equivalent pro-

tection to that given by inorganic phosphate. Three rats (Table II, Nos. 145 to 147) were given a diet similar to Diet 84, except that 2 per cent of the flour was replaced by an equal weight of lecithin (commercial product). The lecithin was dissolved in ether and mixed with the flour in this way, thus ensuring an even distribution of the lecithin throughout the mixture. The ether was then allowed to evaporate at room temperature, and the salt mixture added. The 2 per cent of lecithin was calculated to contain about 79 mg. of phosphorus, making a diet which was comparable in its phosphorus content to Diet 85. The total phosphorus of the diet was found by analysis in duplicate to be 152.4 and 153.2 mg. per 100 gm. of diet.

After 30 days on this diet the rats showed no evidence of rickets either by x-ray examination or microscopically (Figs. 6 and 7). The conclusion is evident, therefore, that phosphorus in the form of lecithin afforded protection equal to that given by inorganic phosphates.

Effect of the Addition of Yeast to Diet 84.

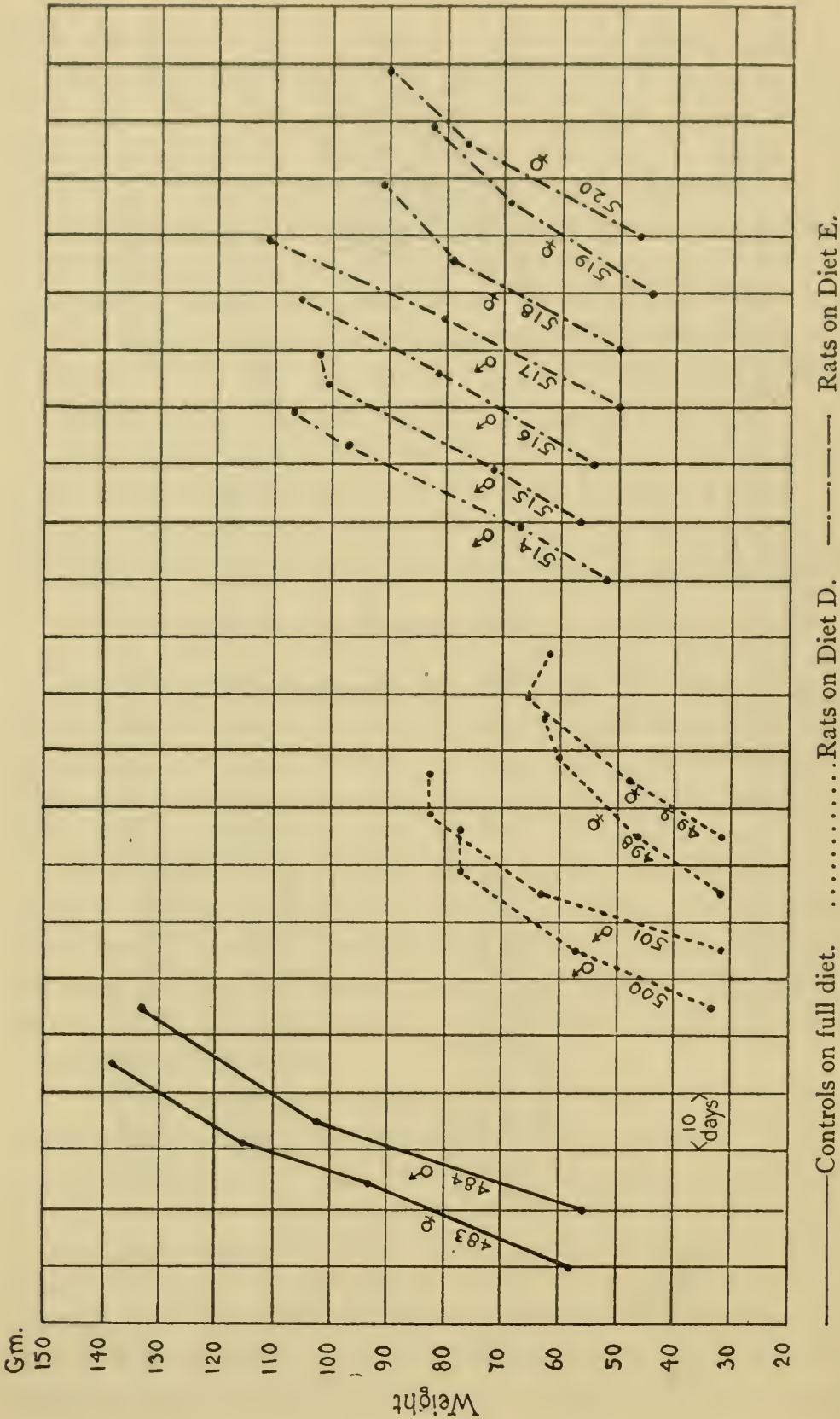
Yeast extract was added to the diet with the double purpose of ascertaining the influence of the water-soluble vitamine and of another phosphorus-containing substance. The preparation used was Harris' yeast vitamine (Osborne and Wakeman (4)) which we found to contain 42 mg. of phosphorus per gm.

In a preliminary experiment about 100 mg. of the extract were measured roughly and sprinkled over each feeding. The rats always ate the yeast avidly as soon as it was placed in the cage. The three rats used for this test (Table III, Nos. 96, 108, and 109) showed no rickets on x-ray examination or section, after 37, 35, and 37 days respectively on the diet.

At the time of the experiment, it was not realized that the phosphorus content of this yeast preparation was as great as it was subsequently shown to be. Therefore, it was necessary to reduce the amount of the yeast extract so as to bring the phosphorus content to, and below the level of protection, and yet supply an adequate amount of water-soluble factor. Data for determining this were available from the work of Osborne and Wakeman (4), who showed that 20 mg. of such a yeast preparation carried ample vitamine to ensure proper growth when the other food constituents were present in

TABLE III.
Effect of the Addition of Yeast Extract to Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet.	Weight.			Radiograph.		Pathological examination.		
				Initial.	Maximum.	Final.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
			days	gm.	gm.	gm.					
96 108	15 17	No. 84 + yeast. (100(?) mg. of Harris' yeast vitamin given daily to rats on basal Diet 84.)	37	37	60	60	22nd	None.	None.*	Slight. Panophthalmitis (right); corneal opacity (left). Moderate keratitis.*	None. "
			35	33	53	52	22nd	"	" *		
109	17		37	29	50	50	22nd	"	" *		"
166 171	28 29	No. 84+0.5 per cent yeast extract containing 21 mg. of phosphorus per 100 gm. of diet.	26	36	46	46	25th	Moderate. Marked.	Slight.*	None. See Table VI.	None.
			26	40	50	50	25th		See Table VI.		
165 167	28 28	No. 84+1.25 per cent yeast extract containing 52 mg. of phosphorus per 100 gm. of diet.	26	33	41	41	25th	Moderate. "	Slight.*	None. See Table VI.	None.
			26	28	50	50	25th		See Table VI.		



TEXT-FIG. 1. Growth of rats on Diets D and E compared with that of controls on a full diet.

adequate amount. There has since accumulated additional evidence that the water-soluble B content of our diets is sufficient to permit of normal growth during the experimental period when the other deficiencies are rectified. Rats on Diets D and E, in which no additional water-soluble vitamine is added, have shown growth closely approximating the normal, at the same time that they were developing rickets (Text-fig. 1). These diets will be discussed under the section on amplified diets.

Two groups were given diets in which 0.5 per cent (Rats 166 and 171) and 1.25 per cent (Rats 165 and 167) of yeast extract replaced equal weights of the flour in Diet 84. In these experiments the extract was mixed evenly through the diet, and not added separately as before. 0.5 per cent of the yeast vitamine was calculated to give 25 mg. of extract per day (the rats each ate about 5 gm. of the total diet per day) and added 21 mg. of phosphorus per 100 gm. of diet. 1.25 per cent gave 62 mg. of extract per day, and 52 mg. of phosphorus per 100 gm. of diet. On these diets all the rats developed marked rickets, as shown by radiograph and section (Figs. 8 and 9).

This would indicate that the water-soluble vitamine may be present in the diet in large amount without any protective effect. A larger indefinite amount of this preparation protected, but here the phosphorus added was sufficient to account for the protection.

Effect of the Addition of Egg Albumin to Diet 84.

The phosphoprotein casein having been shown to afford at least a partial protection, probably dependent upon its phosphorus content, we attempted to improve the nutrition by the addition of a phosphorus-free protein. The phosphorus content of the egg albumin used was negligible, 10 gm. samples not yielding enough ammonium phosphomolybdate precipitate to titrate. 10 per cent of egg albumin (commercial product) was substituted in Diet 84 for an equivalent amount of flour. This addition alone did not strikingly improve the general nutrition. There was also no protective effect. Rat 159 (Table IV) kept on this diet for 26 days showed rickets by radiograph (Fig. 10) and at autopsy (Fig. 11). Rats 160 and 161, also on this diet for 26 days, showed even more severe rickets radiographically and were subsequently used for other work.

TABLE IV.
Effect of the Addition of Egg Albumin to Diet 84.

Rat No.	Litter No.	Diet.	Length of time on diet. days	Weight.			Radiograph.		Pathological examination.		
				Initial. gm.	Maximum. gm.	Final. gm.	Day of diet.	Rickets.	Rickets.	Ophthalmia.	Other lesions.
159	26	No. 84 + egg albumin.									
		Flour.....85.0 per cent.	26	33	43	43	24th	Moderate.	Marked.*	None.	None.
		Egg albumin.....10.0 "									
		Calcium lactate.....2.9 "									
160	26	Sodium chloride.....2.0 "	26	44	50	50	24th	Marked.	See Table VI.		
161	26	Ferric citrate.....0.1 "	24	40	61	61	24th	"	"	" VI.	

Effect of the Addition of Butter and of Butter Fat to Diet 84.

The addition of butter was of particular interest in view of the much discussed rôle of the fat-soluble vitamine in the prevention of rickets.

The conception of rickets as a vitamine deficiency disease was first propounded by Funk (5). The views and experiments of Mellanby (6, 7) in support of this theory have been so widely quoted that no further reference need be made to them.

Subsequent work in general has been unfavorable to Mellanby's conclusion. Harden and Zilva (8) in monkeys, Hess and Unger (9) in infants, Paton and Watson (10) in dogs, Hess, McCann, and Pappenheimer (11) in rats, Mackay (12) and Tozer (13) in kittens, Tozer (14) in guinea pigs, Zilva, Golding, Drummond, and Coward (15) in pigs have all failed to produce typical rachitic lesions on diets deficient in fat-soluble vitamine.

Shipley, Park, McCollum, and Simmonds (16) cite experiments in which a diet adequate in phosphorus, but deficient in fat-soluble A failed to produce rickets. In subsequent papers (17, 18) these authors state that the addition of 10 per cent of butter fat to a rickets-producing diet is ineffective in preventing the development of rachitic lesions, although 3 per cent of the butter-fat carried sufficient fat-soluble vitamine for the promotion of growth and fertility.

The latest publication on this phase of the subject is that of Korenchevsky (19), who concludes that ordinarily a deficiency in fat-soluble A produces in young rats merely an osteoporosis with deficient osteogenesis, but in some cases, namely, when the parents during conception, pregnancy, and lactation have been fed on a fat-soluble-deficient diet, changes more analogous to rickets are produced in the offspring. He concludes, therefore, that vitamine A has a relation to the metabolism of calcium and to the development of rickets.

In our first experiment four rats (Nos. 73, 74, 79, and 80, Table V) were given Diet 84 modified by the substitution of 5 per cent of pasteurized butter for flour. This gave about 0.20 to 0.25 gm. of butter per rat per day. This amount of butter of the same brand completely protected all control animals against keratomalacia and evoked a characteristic rise in the weight curve with cure of keratomalacia in two rats which had been maintained for a long time on a fat-soluble-deficient diet (11). The rats on Diet 84 plus 5 per cent butter, after 32, 43, 45, and 45 days respectively, were killed and found to show marked rickets (Figs. 12 and 13).

Incidentally, it may be mentioned that eye lesions occurred with extreme frequency (over 90 per cent), irrespective of the presence

or absence of rachitic lesions, in rats on Diets 84 and 85, and the modifications described.

In spite of certain work indicating that the fat-soluble vitamine is fairly resistant to heat (Osborne and Mendel (20)), it seemed advisable to make certain that we were giving an excess of this factor by repeating the experiments, using butter which had not been subjected to heat.

To a second series of rats (Nos. 142 to 144, Table V) 0.4 gm. per rat of fresh butter, made every few days in the laboratory from fresh cream, was given daily. This butter contained 20 per cent of whey, and on analysis 2.4 mg. of phosphorus per gm. The 0.9 mg. of phosphorus given daily was thus far below the amount necessary to protect, as shown in the previous experiments. After 28, 30, and 30 days on this diet (Fig. 14) these three rats were killed, and all showed rickets of moderate degree microscopically. While the lesions were not so extreme as those which had been observed in some rats on other diets for a corresponding period, they were quite definite, and there was no evidence of healing or protection.

Moreover, butter fat, though given in amounts carrying ample fat-soluble vitamine, seems to have no curative effect upon rickets already established. Osborne and Mendel (20) found that 6 per cent butter fat carried enough for adequate growth and the prevention of eye lesions. In our experiments 10 per cent of two butter fats, one made from fresh raw (farm) butter and one from the same brand of pasteurized butter as used before, was substituted for an equivalent amount of flour in an amplified diet described below. Rachitic rats Nos. 161 and 190 were given the raw butter fat, and rachitic rats Nos. 160, 167, 169, and 171 (Table VI) the pasteurized butter fat. After 8 to 10 days on this diet these rats still showed marked rickets with no evidence of healing in spite of excellent general health.

The foregoing experiments seem to us to give convincing evidence that the fat-soluble vitamine, as usually defined (see, for example, Steenbock, Sell, and Buell (21)), plays no part in the pathogenesis of rat rickets.

Effect of the Addition of Meat to Diet 84 (Meat and Flour Diet).

Three rats (Nos. 281 to 283) were fed for periods of 33, 39, and 33 days on Diet 84, supplemented by the addition of chopped and dried round steak *ad libitum*. The growth on this diet was normal, and examination of the bones radiographically and microscopically showed a normal structure.

This diet was adequate in both calcium and phosphorus.

Three rats (Nos. 284 to 286) were placed for a period of 35 days upon a diet of dried chopped steak and patent flour, without the addition of sodium chloride, calcium lactate, or ferric citrate. This gave a diet rich in phosphorus, but deficient in calcium. Growth on this diet was poor. The lesions resulting in the ribs resemble rather closely those described in the previous paper on Diet 85C—low in calcium, but containing an excess of phosphate—namely, slight irregularity and widening of the zone of preparatory calcification, defective calcium deposition in the cartilage, and slight excess of osteoid. Marked rachitic deformities were not produced (Fig. 15).

It is interesting to compare these findings with those of Watson (22) in rats maintained on an exclusive meat diet. Watson's rats showed deformities and infractions resembling those of rickets, but microscopically the epiphyseal changes were very slight.

Amplified Rachitic Diet.

In the first paper of this series (2) it was pointed out that Diet 84 is deficient in the character of the protein, in its fat-soluble vitamine content, and in various inorganic constituents. The following diets were devised to remedy these deficiencies.

In a preliminary series rats already rachitic, as shown by radiograph, were placed on Diets 84R, 84R₁, and 84R₂ (Table VI), in which Diet 84 is enriched by the substitution of a more complete protein (egg albumin), butter or butter fat, and a salt mixture complete in every respect except phosphorus (Osborne and Mendel (23)). No tendency to healing of the bone lesions was shown microscopically after 8 to 10 days on this diet. Although the general condition of the animals was greatly improved during this time, as shown by sleekness and amount of body fat, the weight was not greatly increased.

TABLE VI.
Amplified Diets.

Rat No.	Litter No.	Previous diet.	Length of time on previous diet.	Rickets as shown by radiograph.	Amplified diet.					Pathological examination.			
					Diet.	Length of time on diet.	Weight.		Radiograph.		Rickets.	Ophthalmia.	Other lesions.
							Initial.	Final.	Day of diet.	Rickets.			
160	26	No. 84 + egg albumin.	26 days	Marked.	No. 84R.	11	50 gm.	51 gm.	9th	Marked.	Marked.*	None.	None.
161	26	No. 84 + egg albumin.	24	"	" 84R.	10	59	60	9th	"	Very extreme.*	"	"
167	28	No. 84 + 1.25 per cent yeast extract.	26	Moderate.	" 84R ₂ .	11	45	50			Very extreme.*	"	"
169	29	No. 84 with Osborne-Mendel salt mixture.	26	"	" 84R ₁ .	8	26	30			Marked.*	"	"
171	29	No. 84 + 0.5 per cent yeast extract.	26	Marked.	" 84R ₁ .	8	49	50			" *	"	"
190		No. 84.	28	Moderate.	" 84R ₂ .	9	93	96			Very extreme.*	"	"

<i>Diet 84R.</i>		<i>Diet 84R₁.</i>	
Flour	78.0 per cent.	Flour	73.0 per cent.
Osborne-Mendel salt (phosphorus-free)	5.0 "	Osborne-Mendel salt mixture (phosphorus-free)	5.0 "
Calcium lactate	2.0 "	Calcium lactate	2.0 "
Egg albumin	10.0 "	Egg albumin	10.0 "
Butter (pasteurized)	5.0 "	Butter fat (pasteurized)	10.0 "

Diet 84R₂.
Like Diet 84R₁ except that raw butter fat was used.

The diet which is being used at present (Diet D) gives a growth curve which, as has been stated, approximates the normal during about 1 month. The composition of the diet is as follows:

Patent flour.....	80.9	per cent.
Egg albumin.....	10.0	" "
Butter fat.....	5.0	" "
Salt mixture.....	4.1	" "

The salt mixture furnished the following constituents.¹

KCl.....	0.85	gm. per 100 gm. of diet.
Na ₂ CO ₃	0.85	" " 100 " " "
MgCO ₃	0.286	" " 100 " " "
Ca lactate.....	2.000	" " 100 " " "
Ferric citrate.....	0.1	" " 100 " " "
KI.....	0.0002	" " 100 " " "
MnSO ₄	0.00078	" " 100 " " "
NaF.....	0.0024	" " 100 " " "
KAl(SO ₄) ₂	0.00024	" " 100 " " "
<hr/>		
Total.....	4.08962	" " 100 " " "

This salt mixture is based on analyses of the milk of small animals instead of cow's milk, as in the Osborne and Mendel mixture.

Rachitic lesions develop regularly on this diet (Fig. 16). A few of the rats which failed to grow because of infections have shown less deformity than more actively growing rats. This has been noted on other rickets-producing diets, and is in accordance with clinical experience in children. An obvious explanation may be found in the greater phosphorus needs of all tissues in the rapidly growing animal.

In Diet E, 72 mg. of phosphorus were added to the above diet. Owing to the substitution of 15 per cent of non-phosphorus-containing substance for flour, the total phosphorus content was reduced to 145 mg. per 100 gm. of diet. This is an amount which has been shown to be on the border-line of phosphorus requirement. These rats showed excellent growth (Text-fig. 1). Of thirteen rats on this diet,

¹ The reasons for using this salt mixture will be discussed in a forthcoming paper.

eight showed no rickets, four minimal rickets, and only one typical, but moderate, lesions. It is in accord with the explanation offered that the rachitic changes in some of these rats were more marked than on simpler diets with equivalent phosphorus content.

CONCLUSIONS.

1. Casein phosphorus does not completely prevent the development of rickets when substituted in Diet 84 in amount equivalent to a protective dose of basic potassium phosphate.

2. The protection given by lecithin is equivalent to its phosphorus content.

3. The protection given by yeast is at least proportional to its phosphorus content. An amount carrying sufficient vitamine B to promote growth, but insufficient to provide adequate phosphorus, does not prevent rickets.

4. Vitamine A, in the form of butter or butter fat to the amount of 10 per cent of the diet, neither prevents nor cures rickets.

5. The substitution of 10 per cent of egg albumin in Diet 84 improves the nutrition, but does not prevent rickets.

6. The addition of meat to Diet 84, thereby supplying an abundance of phosphorus, promotes normal growth and normal bone formation. A diet consisting solely of meat and flour is inadequate for proper growth, and leads to changes in the bones comparable with those observed on a diet low in calcium, but rich in phosphorus.

7. A diet has been found which contains the necessary food elements for approximately normal growth, and in which the only known deficiency is phosphorus. This leads regularly to the production of rickets.

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EXPLANATION OF PLATES.

PLATE 25.

FIG. 1. Rat 95. 22 days on Diet 84 with the substitution of 10 per cent purified casein. Radiograph showing moderate rickets.

FIG. 2. Rat 95. 22 days on Diet 84 with the substitution of 10 per cent purified casein. The rib shows moderate rickets; early healing (?) (see text). 8 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

FIG. 3. Rat 105. 22 days on Diet 84 with the substitution of 10 per cent purified casein. Radiograph showing slight rachitic changes in the upper epiphysis of the tibia.

PLATE 26.

FIG. 4. Rat 105. Radiograph showing healing of rachitic lesion after 36 days.

FIG. 5. Rat 105. 38 days on Diet 84 with the substitution of 10 per cent purified casein. The rib shows a zone of provisional calcification, three to four cells deep; matrix calcified. Primary spongiosa composed of five or six short stout trabeculae completely ossified, without osteoid margin. Secondary spongiosa poorly developed. Cortex without visible osteoid border. Marrow partly

fatty. No rickets. Osteogenesis inactive. 10 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

FIG. 6. Rat 145. Diet 84 with the substitution of 2 per cent lecithin. Radiograph on the 28th day. No rickets.

FIG. 7. Rat 145. 30 days on Diet 84 with the substitution of 2 per cent lecithin. The rib shows a zone of provisional calcification four to five cells deep. Calcium deposition in matrix normal. Primary spongiosa, extremely delicate, trabeculae missing in places; those present, regular in arrangement and well calcified. Cortex thinned in subchondral region, elsewhere well developed. No excess of osteoid. Slight osteoporosis. No rickets. 5 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 27.

FIG. 8. Rat 166. Diet 84 with the substitution of 0.5 per cent Harris' yeast extract. Radiograph taken on the 25th day. Marked rickets.

FIG. 9. Rat 166. 26 days on Diet 84 with the substitution of 0.5 per cent Harris yeast extract. The rib shows moderate rachitis. Zone of preparatory calcification twenty to thirty cells deep; calcium deposition limited to distal half of cartilage matrix and its prolongations. Dense spongiosa with great excess of osteoid; osteoblasts conspicuous. Osteoid margin about cortex much increased. 3 days decalcification in Müller's fluid. Silver nitrate-Van Gieson stain.

FIG. 10. Rat 159. 24 days on Diet 84 with the substitution of 10 per cent egg albumin. Radiograph showing moderate rickets.

FIG. 11. Rat 159. 26 days on Diet 84 with the substitution of 10 per cent egg albumin. The rib shows typical marked rickets. 9 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 28.

Fig. 12. Rat 79. 32 days on Diet 84 plus 5 per cent pasteurized butter. The rib shows definite rachitic lesions. The zone of provisional calcification projects irregularly and is greatly widened. Calcium deposition defective. Spongiosa dense, composed almost wholly of osteoid with inclusions of cartilage cells. Cortex surrounded by wide osteoid border. Marrow spaces reduced. 2 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

FIG. 13. Rat 80. 45 days on Diet 84 plus 5 per cent pasteurized butter. The rib shows advanced rachitic changes at the chondrocostal junction. 3 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

PLATE 29.

FIG. 14. Rat 144. 30 days on Diet 84 plus 0.4 gm. daily of fresh, unpasteurized butter. The rib shows a zone of preparatory calcification twelve to sixteen cells deep, with several prolongations into the metaphysis. Matrix calcium-free,

except for the central core of spongiosa. Trabeculae of spongiosa broad and abundant, almost wholly osteoid, bordered by conspicuous osteoblasts; osteoid border of cortex increased along entire length of shaft. Moderate rickets. 3 days decalcification in Müller's fluid. Hematoxylin-eosin stain.

FIG. 15. Rat 285. 35 days on meat and flour diet. Zone of preparatory calcification very slightly increased. Calcium deposition limited to matrix between distal two or three cells. Osteoid increased about spongiosa and cortex.

FIG. 16. Rat 498. 26 days on Diet D. Radiograph showing rickets.

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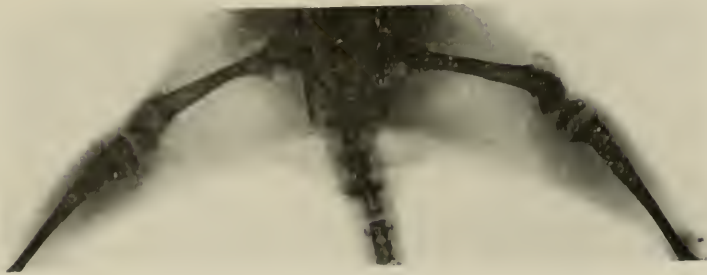


FIG. 1.

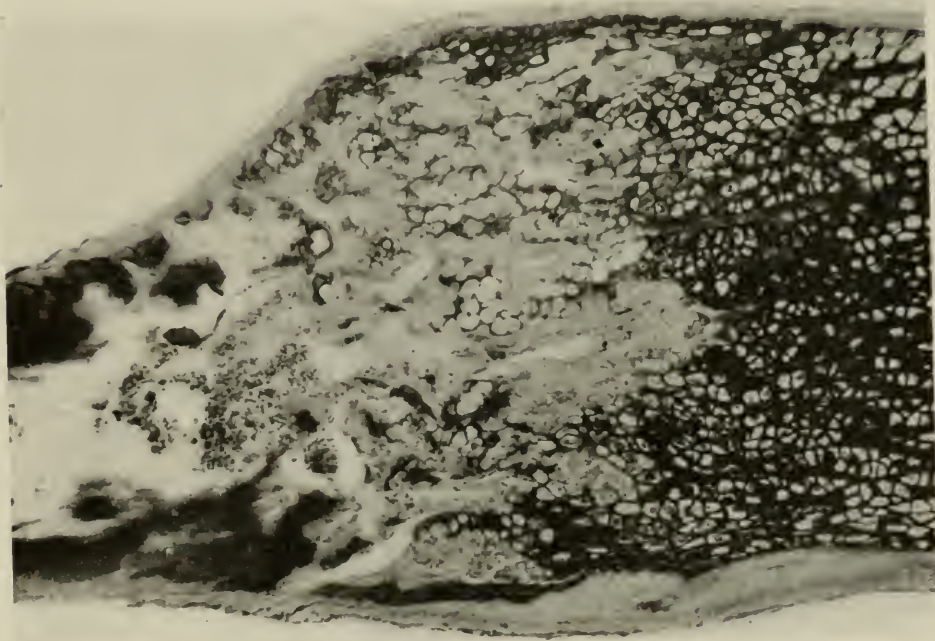
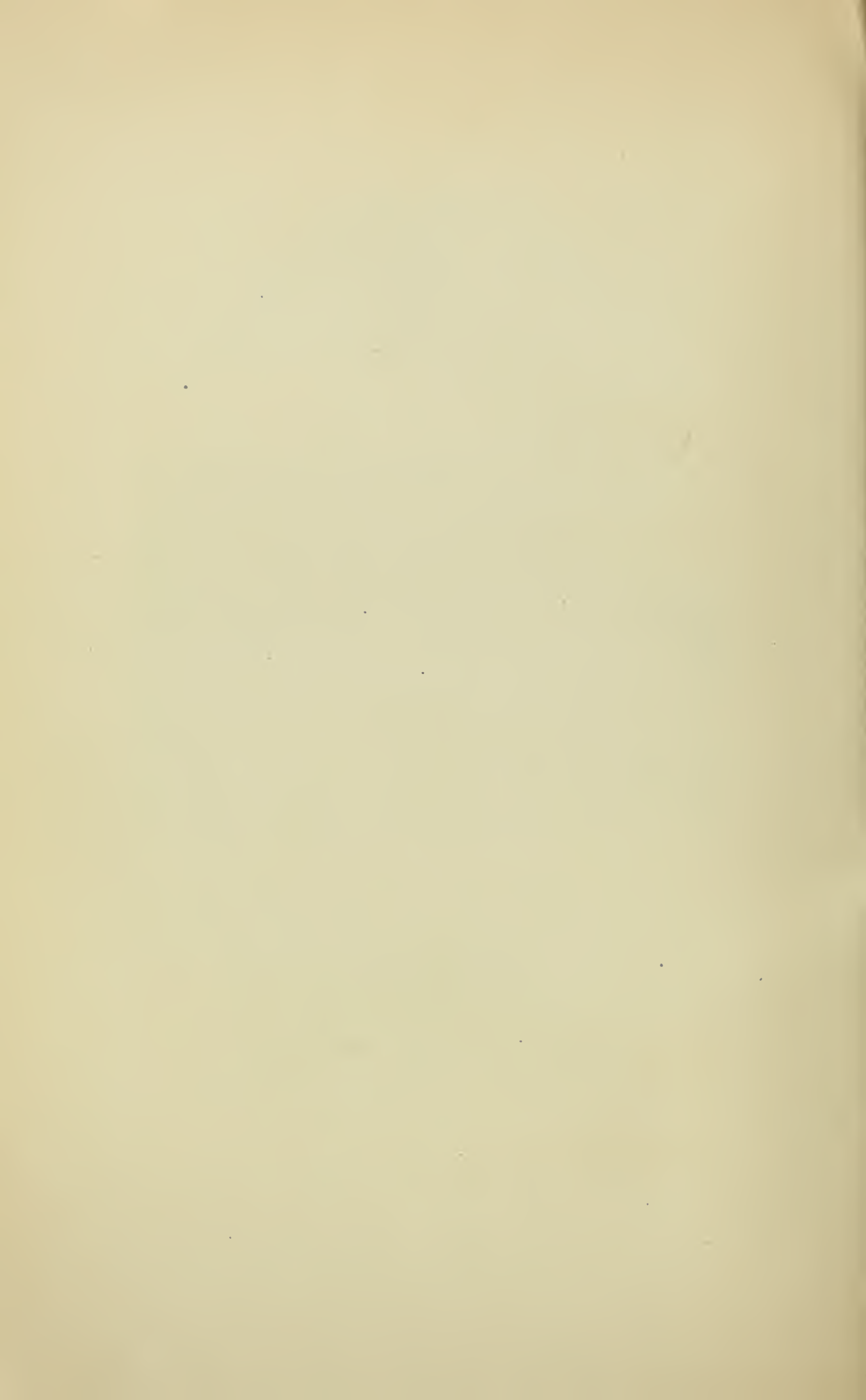


FIG. 2.



FIG. 3.

(Pappenheimer, McCann, and Zucker: Experimental rickets. V.)



466ⁿ



FIG. 4.

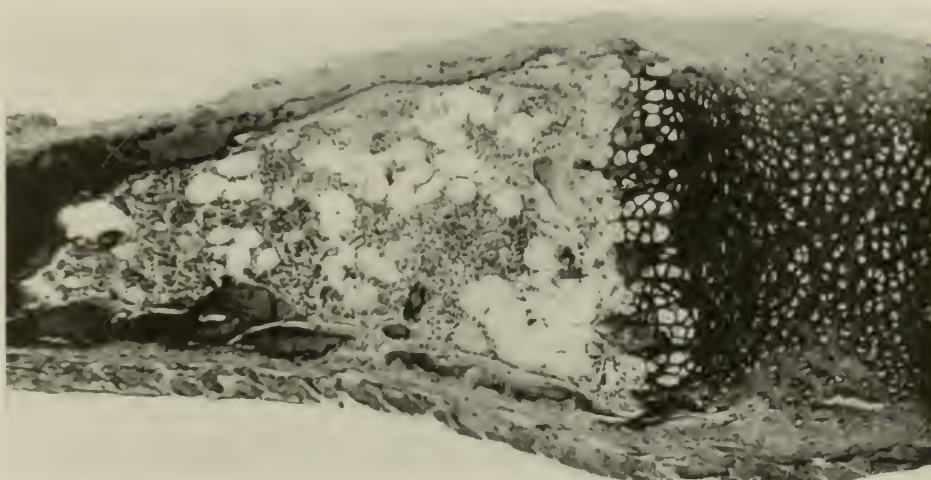


FIG. 5.



FIG. 6.

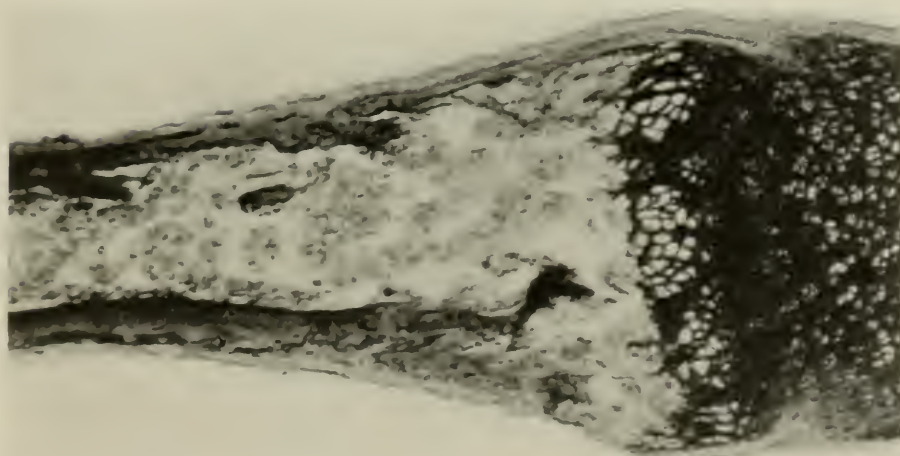


FIG. 7.



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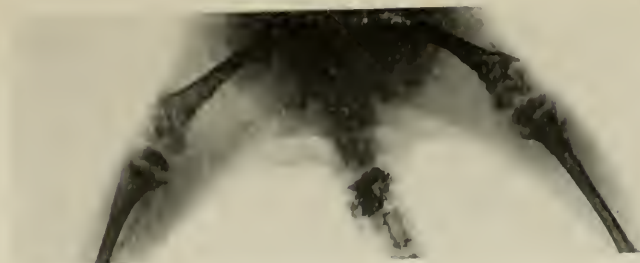


FIG. 8.



FIG. 9.

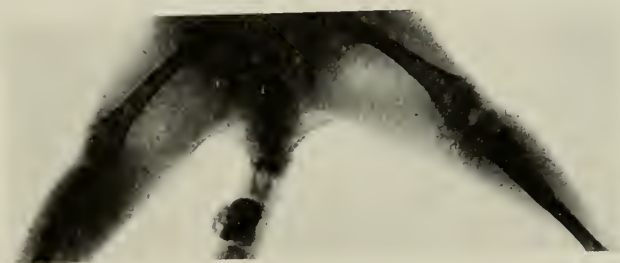


FIG. 10.

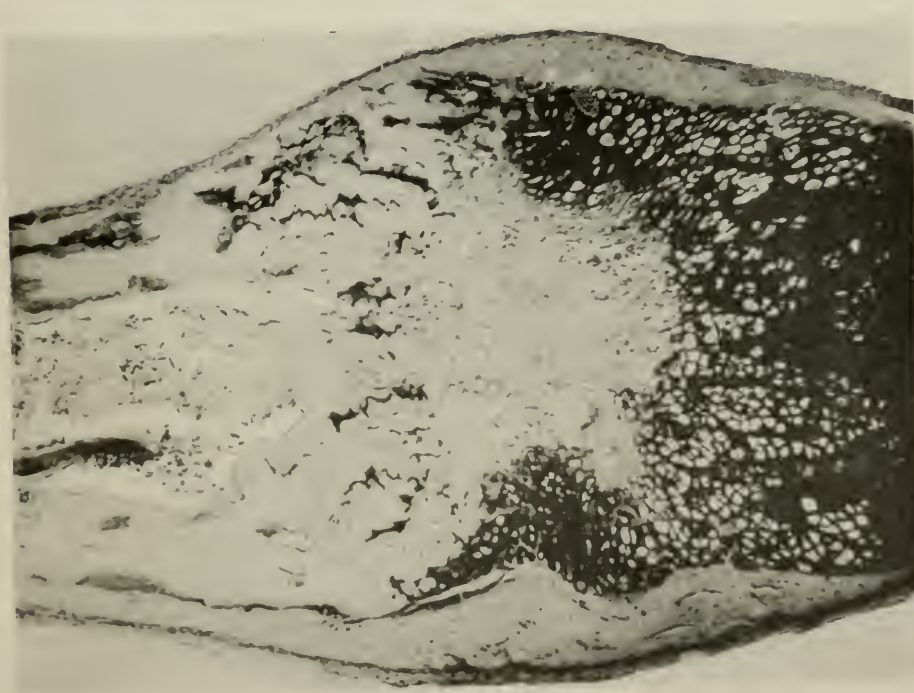


FIG. 11.





FIG. 12.

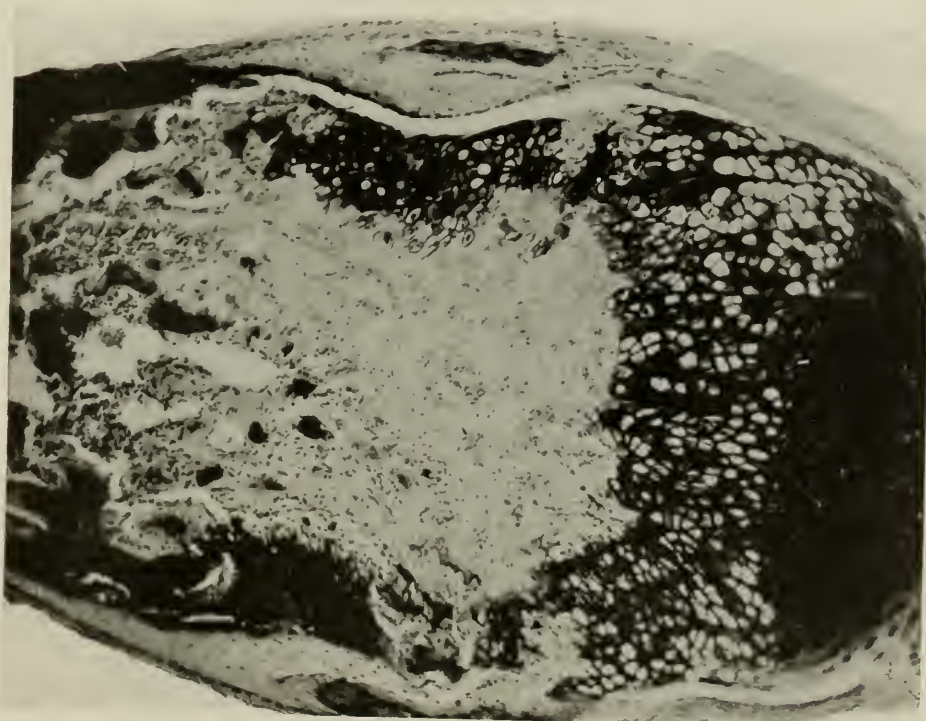


FIG. 13.

(Pappenheimer, McCann, and Zucker: Experimental rickets. V.)

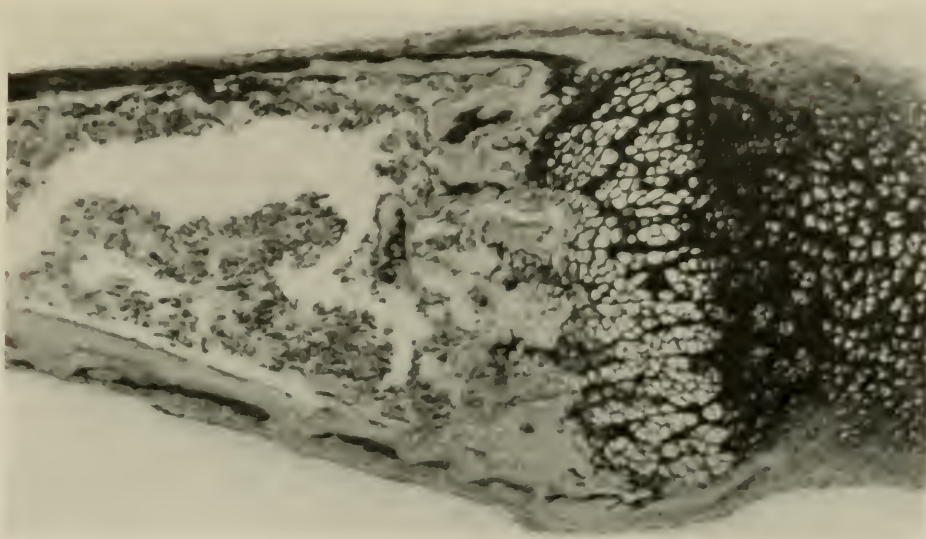


FIG. 14.

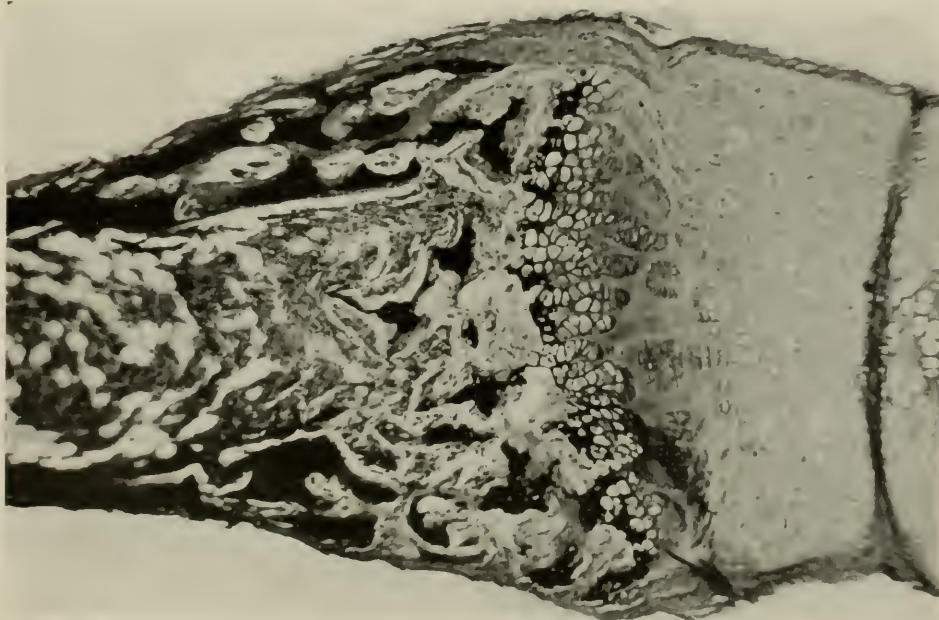


FIG. 15.



FIG. 16.

(Pappenheimer, McCann, and Zucker: Experimental rickets. V.)



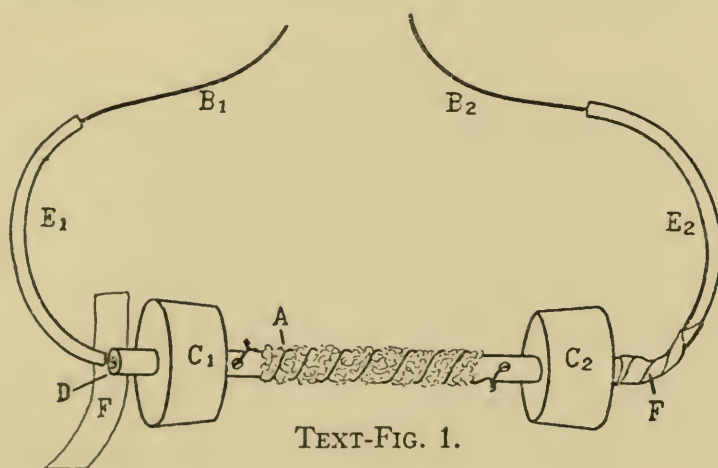
MODIFICATION OF AN IMPROVED ANAEROBE JAR.

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(Received for publication, November 17, 1921.)

The explosion of one of our anaerobe jars after use for a year without accident has led to a slight modification of the apparatus previously described.¹ Reference to Text-figs. 1 and 2 of the article referred to will show the copper wire terminals B_1 and B_2 leading through the rubber stoppers C_1 and C_2 beside the glass tubing D . It has been found that after some months of use the copper wires become corroded where they are in contact with the rubber stoppers, probably due to the interaction of moisture, heat, and some constituent of the rubber stoppers (possibly sulfur). The accident referred to was due to the breaking of one of these corroded wires resulting in the production of a spark just outside the rubber stopper when the electric current was turned on. The corrosion may be avoided by having the copper wires enter the coil through the bore of the glass tube D , joining the nichrome wire A through small holes in the side of the tube within the coil. Although the capillary rubber tubing E_1 and E_2 used for insulation has been found to have no appreciable corrosive action on the copper wires it is further suggested that for B_1 and B_2 one may



use wire insulated with a non-corrosive insulation such as asbestos, thus eliminating the rubber tubing altogether. We also pack the bore of the glass tube with asbestos at either end where the wires pass into it and wrap the joint with insulating tape (F). The modifications recommended are shown in Text-fig. 1.

¹ Brown, J. H., An improved anaerobe jar, *J. Exp. Med.*, 1921, xxxiii, 677.

EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

IV. IMMUNIZING AND TOXIC AGENTS FOUND OCCASIONALLY IN FILTRATES OF TYPHUS-INFECTED TISSUES.

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PLATES 30 AND 31.

(Received for publication, November 7, 1921.)

In a previous article¹ we stated that typhus virus present in tissues of guinea pigs reacting to the experimental disease failed to pass through Berkefeld V and N candles. Reference was also made to the non-filterability of the virus in the blood of infected animals.

The object of this paper is to show that these filtrates, which are free, so far as we can ascertain, from a living, multiplying agent, can occasionally induce in guinea pigs not only the typical lesions of the disease but also immunity to later injections of the active virus.

Method.

The brain and spleen obtained from guinea pigs during the height of experimental infection were chosen as sources of typhus virus, because these organs contain the virus in greater concentration than the blood.^{2,3}

The tissues were disintegrated, thus liberating the virus, and were suspended in Ringer's solution. The method has been described in the previous paper.¹ 1 cc. of the unfiltered suspension was injected intraperitoneally in guinea pigs, which served as controls in demonstrating the activity of the infected tissues. Other portions of the suspensions were passed through tested Berkefeld filters (V and N) and 5 to 10 cc. were similarly injected into thirty-nine test animals. The subsequent disposition of these animals was as follows:

(A) Nine were bled between the 4th and 10th days after inoculation by cardiac puncture and 3 cc. of the defibrinated blood were injected intraperitoneally

¹ Olitsky, P. K., *J. Exp. Med.*, 1922, xxxv, 121.

² Landsteiner, K., and Hausmann, W., *Med. Klin.*, 1918, xiv, 515.

³ Weil, E., Breinl, F., and Gruschka, T., *Wien. klin. Woch.*, 1921, xxxiv, 459.

into each of eighteen normal animals. This was for the purpose of disclosing any living microorganism in the filtrates.

(B) Twenty were observed for periods up to 16 days for clinical signs, especially early or late febrile reactions.

(C) Nine of Group B were reinjected with active blood from 10 to 16 days after the first inoculation. This was done to determine the immunizing power of filtered virus.

(D) Finally, ten were killed from 4 to 7 days after inoculation in order to note any changes produced in the tissues.

RESULTS.

The following is a summary of the results of the experiments.

Activity of the Virus Employed.—All the guinea pigs which were injected with the suspensions of unfiltered typhus-infected tissue showed, after an average incubation period of 7 to 8 days, a febrile reaction lasting, as a rule, for 8 days. After recovery, the animals remained resistant to later injections of active blood. Those killed at the height of reaction exhibited the characteristic histopathological changes of experimental typhus fever. With the blood obtained in this stage, a disease of similar type was produced in normal animals. No secondary or concurrent infections were found. The disease was, therefore, typical of experimental typhus fever in guinea pigs, as described in detail in another communication.⁴

Absence of Living Organisms.—The blood of guinea pigs which were inoculated with filtrates failed in each instance to induce in normal animals the typical fever observed in the controls. It must be concluded that the filtrates, in view of the failure to reproduce the disease by transmission, were free from a living, multiplying agent.⁵

Temperature Reactions.—Of the twenty guinea pigs inoculated with filtrates, fifteen revealed no disturbance of temperature. Of the remaining five, all showed a rise in temperature, above 40°C. (104°F.), for 1 to 3 days, first noted from 2 to 5 days after the inoculation.

Immunity.—Of the nine guinea pigs inoculated with filtrates and reinjected after 10 to 16 days with active typhus virus, six reacted typically to the second injection, and two showed a mild fever, lasting

⁴ Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

⁵ Experiments demonstrating the non-filterability of the typhus virus have been described in the previous paper.¹

for 4 days, instead of 8 days as in the controls, which on transmission proved to be the familiar experimental typhus, while one was not affected. Thus, the filtrates induced no immunity in six, a partial immunity in two, and a complete immunity in one of the animals.

Production of Lesions.—In four of ten guinea pigs inoculated with filtrates and killed 7 days after injection, the following lesions were found.

Macroscopic Examination.—The organs were normal in appearance, with the exception of the spleen, which was slightly enlarged and darker than is usual. In two guinea pigs a few small discolored areas, resembling the spots of a petechial rash, were seen after stripping the corium of the skin.

Microscopic Examination.—The central nervous system showed no meningeal involvement, except in its blood vessels. Focal accumulations of macrophages, or mononuclear cells, were noted about the capillaries and arterioles, which at some points were sufficient to form a distinct nodular mass giving rise to a periarteriolitis nodosa (Fig. 1). The endothelium of the vessels, often swollen, was necrotic in some portions and proliferated in others. Occasionally an arteriole or capillary was noted to contain occluding thrombi. The gray matter of the brain, especially that of the midbrain, contained a few small localized hemorrhages. The vessels here revealed lesions similar to those found in the vessels of the meninges. Nodules were seen, always in proximity to the vessels, which consisted mainly of macrophages, or mononuclear cells, and a few polymorphonuclear cells (Fig. 2).

The heart tissue revealed no visible changes except for a few localized hemorrhages and the vascular lesions described above (Figs. 3 and 4).

The changes in the spleen were such as to obscure the vascular lesions. There were noteworthy congestion and leucocytic infiltration. The lymphoid follicles were enlarged and at the center were a number of macrophages replacing to some extent the lymphoid cells.

The skin in two of the guinea pigs exhibited in the deeper layers of the corium small localized hemorrhages and the vascular lesions and nodules described above (Fig. 5).

The lesions found in these animals are characteristic of experimental typhus fever in the guinea pig.⁴

To summarize the results, it appears that in a small number of instances other effects than typhus are produced by the inoculation of filtrates from typhus-infected tissues. In five of twenty guinea pigs, a rise in temperature occurred which lasted from 1 to 3 days. One of nine guinea pigs remained immune and two others of this

series responded with a mild reaction, after a test injection with active virus. Finally, four of ten animals showed the characteristic lesions of the experimental disease.

DISCUSSION.

We have already demonstrated¹ that the typhus virus present in the tissues of guinea pigs at the height of their reaction to inoculation is not filterable through tested Berkefeld filters. Hence effects produced by means of filtrates of infected tissues cannot be attributed to a living, multiplying agent. The possibility of the presence of subinfective quantities of the virus in filtrates producing the results can be eliminated by the fact that whereas only infinitesimal amounts of infected brain tissue (0.005 gm.)² are necessary to induce the experimental infection, we have employed almost one-half of the amount of entire filtrate obtained from suspensions of all the brain tissue from 300 to 350 gm. guinea pigs. Furthermore, in our experience¹ transmission of the virus from animal to animal by means of filtrates has failed.

The experiments reported in this paper demonstrate that such filtrates can produce occasionally an early and short febrile reaction, immunity to later injections of active virus, and lesions indistinguishable from those of the typical experimental disease. From this we infer that a specific substance may be present in the tissues of infected guinea pigs and be occasionally obtainable in filtrates.

Nicolle and his associates⁶ have maintained that the typhus virus is filterable for the reason that infrequently monkeys which have been inoculated with filtrates of blood, or of lice harboring the virus, have shown no definite temperature reaction, but remained resistant to a later injection of active virus. The experiments above described demonstrate that such occasional immunity following inoculations of filtrates depends on some other factor than a living, multiplying agent.

⁶ Nicolle, C., Conor, A., and Conseil, E., *Compt. rend. Acad.*, 1910, cli, 685; 1911, cliii, 578. Nicolle, C., Blanc, G., and Conseil, E., *Arch. Inst. Pasteur Tunis*, 1914, ix, 84.

CONCLUSION.

In the filtrates of typhus-infected tissues of guinea pigs can be occasionally found a substance which produces in these animals thermic reactions, lesions characteristic of experimental typhus, and, still less frequently, immunity to later injections of active virus. The general indications are that this substance is not a living organism.

EXPLANATION OF PLATES.

PLATE 30.

FIG. 1. Section of the brain of a guinea pig inoculated with a filtrate of typhus-infected tissue. The vessels of the meninges show the perivascular accumulation of macrophages of the nature of a nodular formation and occlusion by thrombus of other vessels. A small nodule can be seen in the gray matter near the surface. $\times 240$.

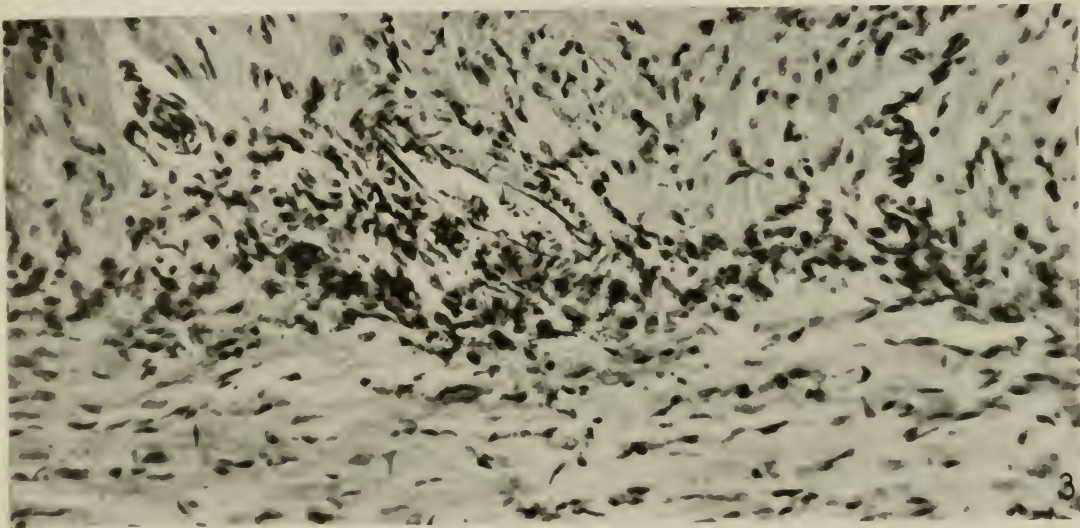
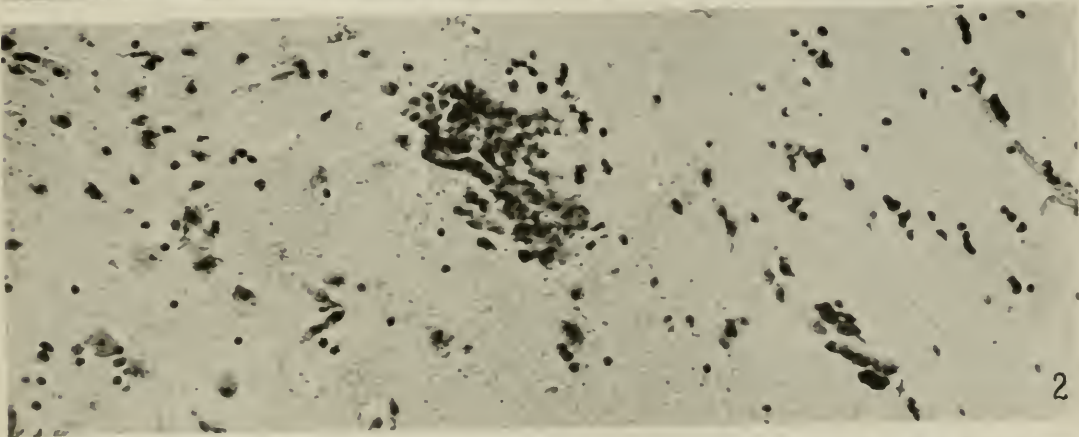
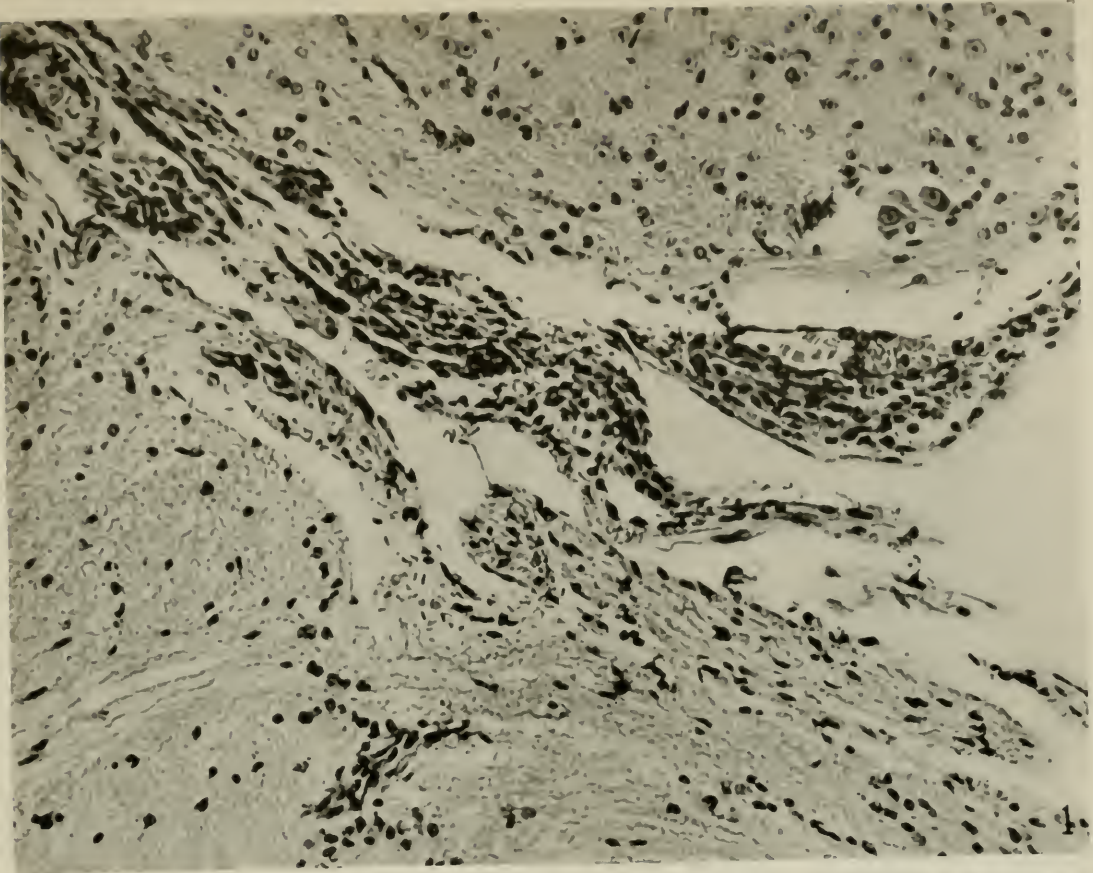
FIG. 2. Another section of the same brain. A distinct nodule in the gray matter can be observed. $\times 240$.

FIG. 3. Section of heart tissue from the same guinea pig, showing vascular lesions similar to those in the brain. $\times 240$.

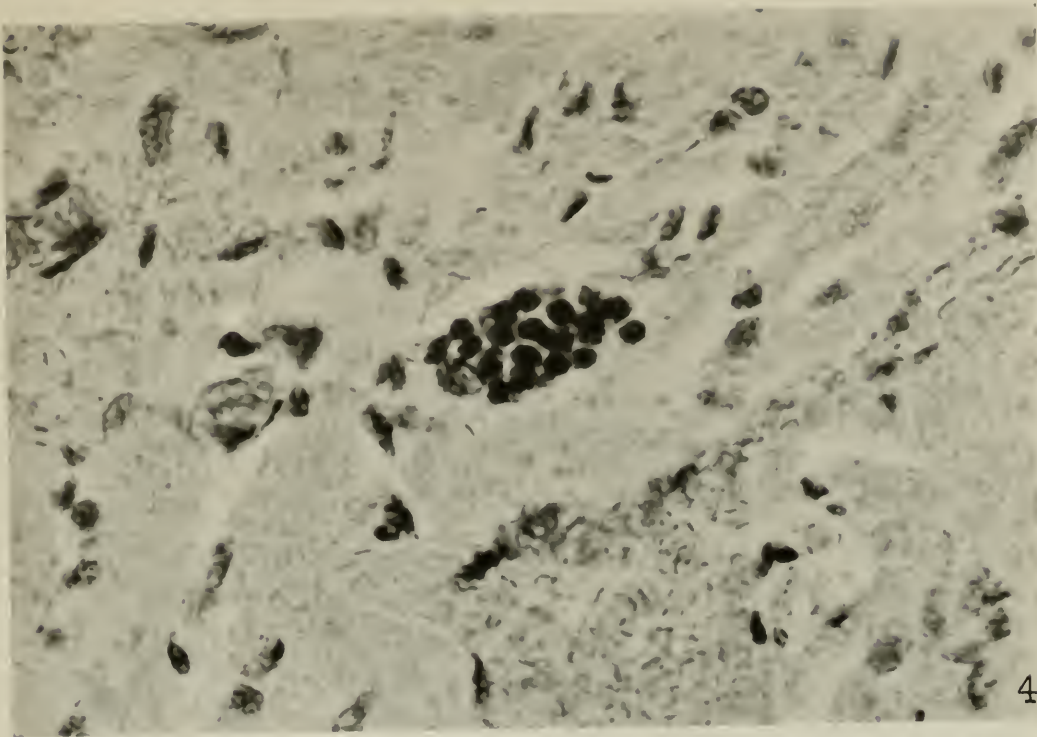
PLATE 31.

FIG. 4. Another section of heart tissue. A nodule under higher magnification to show its structure. $\times 560$.

FIG. 5. Section of the deeper layers of the skin from the same guinea pig. At the edge of the section, toward the epithelial surface, is a small nodule indicated by an arrow; in the deeper muscular layer is a pronounced vascular lesion similar to that noted in Fig. 1. $\times 240$.



(Olitsky: Etiology of typhus fever. IV.)



STUDIES ON X-RAY EFFECTS.

X. THE BIOLOGICAL ACTION OF SMALL DOSES OF LOW FREQUENCY X-RAYS.

BY WARO NAKAHARA, PH.D., AND JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 32 AND 33.

(Received for publication, November 2, 1921.)

Attempts have been made in this laboratory to compare the biological action of the soft or low frequency and the hard or high frequency x-rays, but the impossibility of comparing doses of the two types of rays with our present apparatus and measuring devices has rendered the results of these studies of little value. However, by varying the doses of the soft and hard rays there is one point which seems definite; namely, that with the softer rays it is possible to induce an apparent stimulation of the lymphoid cells which is preceded by only a very short and transitory period of depression.¹⁻⁴ With the harder rays the stimulation phase is less pronounced, if present at all in the dosages employed by us. Russ, Chambers, Scott, and Mottram⁵ have succeeded in bringing about an increase in the circulating lymphocytes of rats by repeated small exposures to x-rays described by them as being of "medium" or "medium soft" quality. Our first satisfactory results were obtained with the tube operated at a spark-gap of about $1\frac{1}{2}$ inches. Later experiments with a spark-gap of about $\frac{7}{8}$ inch gave a more uniform and pronounced reaction.

The present study has to do with the effect of still softer x-rays than those used in previous experiments. For the generation of these

¹ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

² Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

³ Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83.

⁴ Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

⁵ Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

rays a special water-cooled tube has been devised with a window of thin glass which will allow the passage of rays usually held back by the thicker glass of the standard tubes.⁶

Production of Lymphocytosis.

The following experiments were planned to determine the time of exposure to the soft rays necessary for the production of a maximum stimulation of the lymphoid system of mice.

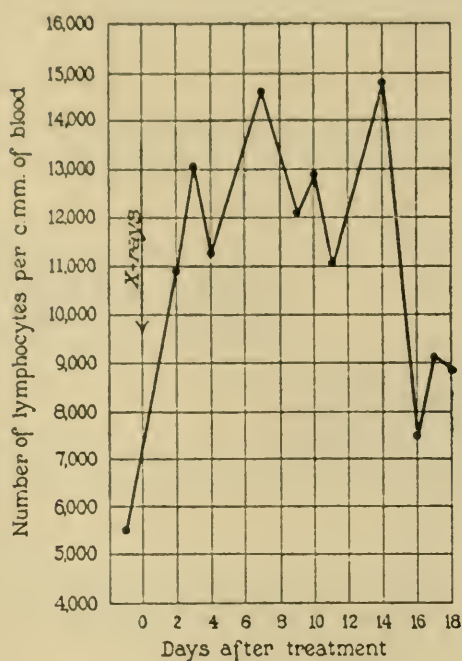
Experiment 1.—Forty-five white mice were divided into nine groups of five mice each. The groups were exposed to x-rays for $\frac{1}{2}$, 1, $2\frac{1}{2}$, 4, 5, 10, 20, 30, or 60 minutes respectively. The special tube described above was used with $\frac{1}{2}$ inch spark-gap, 11 milliamperes, and 6 inches distance, for the treatment. A cardboard was placed over the animal container for protection against the heat. Blood counts were made on all of the animals before and 1 week after the treatment. The mice given the 1 minute exposure showed a definite lymphocytosis, but no consistent change was noted in the blood picture of the other groups.

Experiment 2.—Blood counts were made on thirty normal white mice and they were then exposed to x-rays given by the special tube with the governing factors the same as in Experiment 1, the length of the exposure being 1 minute. Counts were made at intervals afterwards. The changes in the lymphocytes are shown in Text-fig. 1 by a composite curve and the results for the individual animals in Table I.

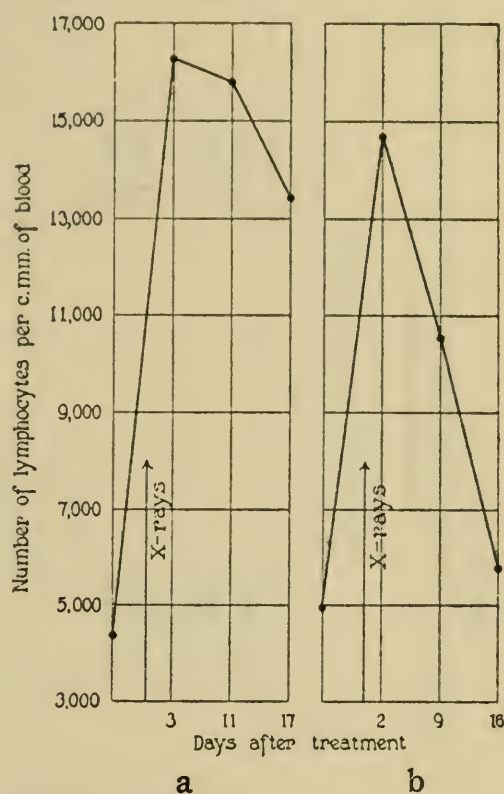
The counts showed that there was no definite alteration in the absolute number of polymorphonuclear leucocytes, but the lymphocytes were increased to a level considerably above that of the initial count. By the 2nd day these latter cells had almost doubled their number and continued to increase with some variations until the 14th day, after which they subsided but were still above their initial level on the 18th day. There was considerable variation in the extent and duration of the reaction in individual mice, as shown in Table I and in Text-fig. 2. In only two animals was there a drop in the lymphocytes at the time of the first count after the x-ray treatment. One of these, however, showed a reaction later with an increase of over 30 per cent, but in the other no stimulation was observed. It should be noted here that none of these counts were made soon enough after the

⁶ We wish to acknowledge our indebtedness to the Research Laboratory of the General Electric Company for the design and construction of this tube.

x-ray treatment to demonstrate the amount of depression preceding the stimulation, for this point has been adequately dealt with in previous publications.⁷ The increase in number of circulating lymphocytes after this dose of x-rays is of greater magnitude and more constant than that previously induced by this agent.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Composite curve of lymphocyte counts on thirty-five mice before and after an exposure to low frequency x-rays.

TEXT-FIG. 2, *a* and *b*. (*a*) Curve of lymphocyte counts on Mouse 23 in Table I. (*b*) Curve of lymphocyte counts on Mouse 12 in the same table.

⁷ In a recent article Mottram and Russ (Mottram, J. C., and Russ, S., *J. Exp. Med.*, 1921, xxxiv, 271) have objected to our use of the term "stimulating" doses of x-rays in describing the effect of this agent on the lymphoid tissue. They object to this term on the basis of the fact that the stimulation is preceded by a short period of depression. This point was noted in our first publication on the subject¹ and has been repeatedly referred to in subsequent articles.^{4,9} The fact that with a suitable small dose of x-rays a stimulation of the lymphoid tissue is produced with only a slight and transitory depression, while with larger doses a more marked and lasting depression is induced with no subsequent stimulation, seems to us to justify the use of the expression.

TABLE I.

Mouse No.	Count before treatment.	Count after treatment.										
		No. of days after x-ray treatment.										
		2	3	4	7	9	10	11	14	16	17	18
1	6,018				15,908				13,250			
2	6,541				11,105				17,713			8,430
3	5,897				14,455							
4	5,899				16,210				14,446			
5	5,526				15,463				14,744			
6	5,823	11,041				12,252				7,091		
7	4,617	7,658				16,532				11,645		
8	4,336	12,070				10,603				8,455		
9	6,585	17,070				13,650				9,423		
10	4,239	10,972				11,766				5,471		
11	4,288	7,604				10,567				5,816		
12	4,998	14,734				12,786				7,005		
13	7,104	7,070				10,472				6,808		
14	5,477	7,406				10,332				6,520		
15	5,185	12,878										
16	6,110		16,122				12,800				8,888	
17	6,113		12,775				16,861				7,133	
18	4,215		9,435				12,890				6,008	
19	4,909		12,002				7,338				5,360	
20	4,790		7,007				9,495				9,231	
21	3,714		12,064				9,304				8,232	
22	4,145		10,989				12,543				9,382	
23	4,425		16,261				15,814				13,209	

24	6,203	18,252	7,527	14,628	12,105	12,978	11,037	15,038	7,581	9,245	12,124	9,897
25	7,155	15,352	7,027			17,055	8,975				12,836	6,675
26	3,594		8,815				7,196					6,878
27	9,313		3,767				7,986					10,178
28	4,428		12,452				12,870					
29	5,264		15,300				15,082					
30	4,404		13,167				10,277					8,571
31	3,974		16,480				17,274					14,796
32	8,330		16,787				12,894					9,893
33	7,329		11,851				8,518					5,092
34	6,175											
35	5,635											
Average.....	5,507	10,850	13,026	11,317	14,628	12,105	12,978	11,037	15,038	7,581	9,245	8,934

The counts before treatment were made a number of days in advance so that no animal had more than one count in a week. For example, on the group of mice counted 2 days after treatment, the pretreatment count was made 5 days before, thus allowing a week between counts.

Histological Changes.

The material for this study was collected from twenty-six mice which were carried as a parallel series to the above experiment in which blood changes were observed. After exposure to x-rays (special tube, spark-gap $\frac{1}{2}$ inch, milliamperes 11, distance 6 inches, time 1 minute) these animals were killed in groups at intervals of 24 hours, 4 days, 7 days, 10 days, and 14 days. The spleen, lymph nodes, bone marrow, thymus, thyroid, liver, kidney, suprarenal, ovary, and testis were taken for examination. Carnoy 6-3-1 solution was used for fixation and the sections were stained with Heidenhain's iron-hematoxylin for mitotic figures or with Ehrlich's acid hematoxylin and eosin.

Lymphoid Organs.—The changes in the spleen and lymph nodes were practically identical and will be described together. 24 hours after treatment there was no demonstrable change in the lymphoid organs. At the 4 day period numerous mitotic figures were found in all of these organs, a condition which our previous work indicates is associated with the increased production of lymphoid cells. This condition was found equally as pronounced in the animals killed on the 7th day after treatment. The organs from eleven mice were examined for these two periods and without exception this increase in proliferation was observed. The animals killed on the 10th and 14th days after treatment showed that the proliferative activity had subsided to about the normal rate.

Suprarenal Glands.—The suprarenals were found at autopsy to be distinctly reddish in color and somewhat enlarged. Microscopic examination showed the sinus-like spaces between the cortex and medulla to be much distended with blood, and this dilatation extended to the capillaries between the cortical cell columns, separating these columns by a wide margin (Figs. 1 and 2). The sinusoids of the medulla showed little if any modification. There was no sign of hemorrhage or of necrosis of the suprarenal tissue in any of the specimens examined. This vascular change was observed in varying degrees in all of the mice examined except those killed 24 hours after the treatment. In addition to this engorgement of the capillaries, a proportion of the animals killed between 7 and 14 days after the

x-ray treatment showed a pronounced perivascular infiltration in the cortex along the fibrous capsule and often extending down to the zona reticularis along the cortical cell columns.

Other Organs.—There was no histological evidence of changes in the other organs examined after the x-ray treatment. Even the germ cells of the fourteen ovaries and twelve testes examined, cells well known to be extremely sensitive, showed no damage which could be detected microscopically (Figs. 3 and 4). In a few instances the interstitial tissue of the testis was found to be hypertrophied. Distinct perivascular lymphoid infiltration was observed in a number of the livers and kidneys examined, but these findings were not uniform enough to be of great importance. This condition is also found occasionally in normal animals.

Effect on Resistance to Transplantable Cancer.

In previous publications it has been shown that a small dose of x-rays sufficient to stimulate the lymphocytes will increase the resistance of mice to transplanted cancer.^{1,8,9,10} Russ, Chambers, Scott, and Mottram⁵ have shown that the same condition holds for rats. As noted above, a more pronounced reaction of the lymphoid tissue is induced by the treatment outlined here than by the dosage previously used. It is of interest, therefore, to determine the effect of the very soft rays on the resistance to cancer.

Experiments 3 to 9.—Normal white mice of about the same age and size were exposed to a dose of x-rays governed by the same factors as those used in Experiment 2, with the special x-ray tube described above. These animals were inoculated in groups from 3 to 14 days after the treatment, with a graft of a transplantable tumor (Bashford Adenocarcinoma No. 63) along with a suitable number of controls. The results of seven such experiments are given in Table II.

From these experiments it is seen that no immunity is evident when the inoculation is made as early as 3 days after the x-ray treatment, a result which corresponds with the earlier experiments in which the standard Coolidge tube was used. Among 86 mice inoculated from 7

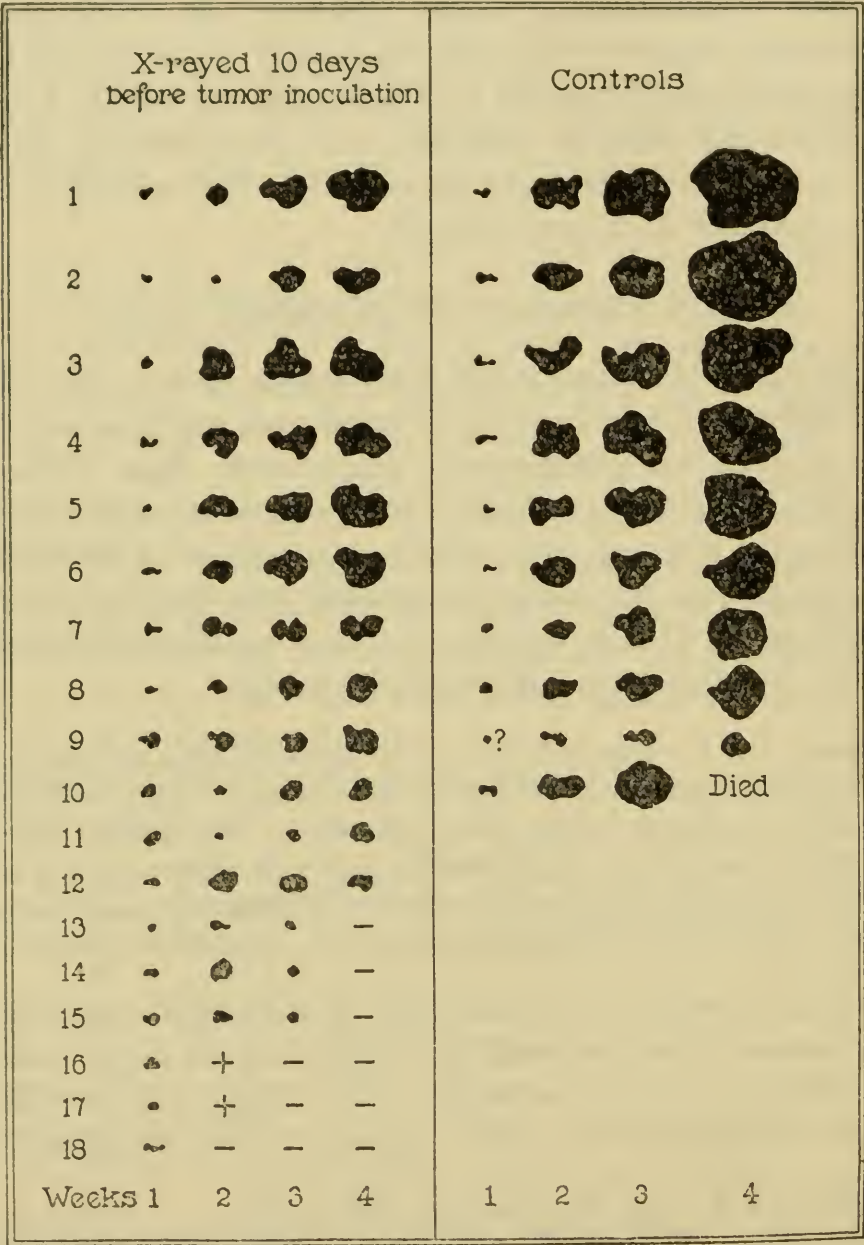
⁸ Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35.

⁹ Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

¹⁰ Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

TABLE II.

Experiment No.	Interval between x-ray exposure and tumor inoculation.	Immunity in x-rayed animals.	Immunity in control animals.
	days		
3	3	0.0 per cent (9 mice).	0.0 per cent (10 mice).
4	7	30.0 " " (10 ").	11.1 " " (9 ").
5	7	28.5 " " (17 ").	9.9 " " (11 ").
6	10	37.5 " " (8 ").	0.0 " " (10 ").
7	10	33.3 " " (18 ").	0.0 " " (10 ").
8	10	57.8 " " (19 ").	10.0 " " (10 ").
9	14	20.0 " " (10 ").	0.0 " " (7 ").



TEXT-FIG. 3. Experiment 7. The rate of growth of Bashford Adenocarcinoma No. 63 in mice given a small dose of low frequency x-rays 10 days before inoculation, contrasted with the rate of growth in untreated mice.

to 14 days after the treatment, 34.4 per cent were immune, while in 57 control mice inoculated with the same tumor there were only 5.1 per cent immune. The largest proportion of resistant mice was found among the animals treated 10 days after inoculation, with 42.8 per

X-rayed 10 days before tumor inoculation				Controls			
1	•	•	•	•	•	Died	
2	•	•	•	•	•	Died	
3	•	•	•	•	•	•	
4	•	•	•	•	•	•	
5	•	•	Died	+	•	•	
6	•	•	•	•	•	•	
7	+	•	•	•	•	•	
8	+	•	•	•	•	•	
9	•	•	—	•	•	•	
10	•	•	—	+	—	—	
11	•	+	—				
12	+	—	—				
13	+	—	—				
14	+	+	—				
15	+	+	—				
16	+	—	—				
17	+	—	—				
18	+	—	—				
19	+	—	—				
Weeks	1	2	3	1	2	3	

TEXT-FIG. 4. Experiment 8. The rate of growth of Bashford Adenocarcinoma No. 63 in mice given a small dose of low frequency x-rays 10 days before inoculation, contrasted with the rate of growth in untreated mice.

cent in 45 mice against 3.3 per cent in 30 control mice. Text-figs. 3 and 4 show that the rate of growth of the tumor is slower in the treated animals than in the controls, and that in a proportion of the treated mice tumors after a period of growth are absorbed, a condition which rarely occurs with this tumor in normal mice.

The Absorption Coefficient of X-Rays Produced by a Voltage Measured by a $\frac{1}{2}$ Inch Spark-Gap.¹¹

It is of considerable interest in the light of the above experiments to arrive at some idea of the amount of penetration of the soft rays. The very soft rays produced by a voltage measured by a $\frac{1}{2}$ inch point spark-gap were found to be practically homogeneous with a mass absorption coefficient equal to 3.4 for water. This value includes the mass-scattering coefficient usually taken as 0.2.

The value was measured photographically. A series of small areas of a single film was exposed to the rays for various times. A second series on the same film was exposed for various times to rays which had passed through layers of water of various thicknesses. After development the areas of the second series were matched for blackness against those of the first series and the relative intensities of the rays transmitted by the water thus discovered. A preliminary experiment gave the value for the absorption coefficient which was used to calculate the exposures through various depths of water necessary to give equal degrees of blackening. Another film was made accordingly and the exposed areas were found to be of uniform density. It will be noticed that, according to the quantum law, the wave-lengths produced by a $\frac{1}{2}$ inch gap are in the neighborhood of the K characteristic absorption discontinuities of the silver and bromine of the plate. In spite of this it can be shown that the constant value found for the absorption coefficient is a proof of homogeneity.

The mass absorption coefficient of these rays in animal tissues may be assumed to be somewhat smaller than for water. A mouse skin weighing 0.2 gm. per cm. should stop less than one-half of the rays.

The relative amount penetrating various depths of water when this water-cooled tube is operated at $\frac{1}{2}$ inch spark-gap is as follows:

Depth.	Intensity.
cm.	
0	1.0 (taken as standard).
$\frac{1}{4}$	0.43
$\frac{1}{2}$	0.18
$\frac{3}{4}$	0.076
1	0.032
$1\frac{1}{4}$	0.0133
$1\frac{1}{2}$	0.0056

¹¹ We are indebted to Dr. Harry Clark for the measurements recorded.

DISCUSSION.

The comparison of the biological effect of the hard and soft rays is a matter of considerable interest at the present moment but no really satisfactory standard of measurement is available. Such comparisons, therefore, must await a further understanding of the complex nature of the biological changes and the underlying factors bringing about these changes.

As far as our present knowledge extends the only known physical or chemical change induced by x-rays depends on the power of this agent to ionize. If ionization be the underlying factor responsible for the biological changes, it is necessary to determine why some types of animal cells are so profoundly affected in the absence of demonstrable changes in other cells. However, the solution of such problems as this must await further development in biophysics.

In this communication evidence is presented of a biological change induced by a small dose of the very soft x-rays; namely, a stimulation of the lymphoid cells preceded in all probability by a small amount of destruction. We have not been able to induce a reaction of this nature with the harder rays given in varying dosage. Until it is determined that the dosage is the same it will not be possible to say whether this apparent difference in biological action of the soft and hard rays is a real one.

Considering the smallness of the dose, the fact that 57 per cent of the x-rays used here is absorbed in the first $\frac{1}{4}$ cm. of tissue and that only 3.2 per cent penetrated to the depth of 1 cm. strongly suggests that the changes in the lymphoid organs are not the result of the direct action of the x-rays. The fact also that the deeper nodes react as much as the more superficial ones strengthens this idea. The significance of the changes in the suprarenals can only be determined by further study. It is noteworthy that the testicle and ovary, organs supposed to be extremely sensitive, were unaffected by this treatment.

The virulence of the strain of tumor used to test the resistance of the mice was such that the so called natural resistance was almost completely obviated, a fact which renders the result of the inoculation of the treated mice the more striking.

SUMMARY.

A study has been made of the biological effect of a small dose of soft x-rays given off by a special water-cooled tube with a window of thin glass, operated at $\frac{1}{2}$ inch spark-gap and 11 milliamperes. Mice exposed for 1 minute show 2 days later in the blood an increase in the number of lymphocytes and in the lymphoid organs an increased number of mitotic figures. There occurs also a marked dilatation of the vessels of the suprarenals, particularly between the cortex and medulla. The latter condition did not appear until after 24 hours and was still present 14 days after the treatment. No change was detected in other organs.

Mice treated in this way showed a high degree of resistance to cancer transplants. The amount of resistance varied with the time of the inoculation after the treatment. The resistance was not increased before 3 days after and was at its highest point 10 days after the treatment.

EXPLANATION OF PLATES.

PLATE 32.

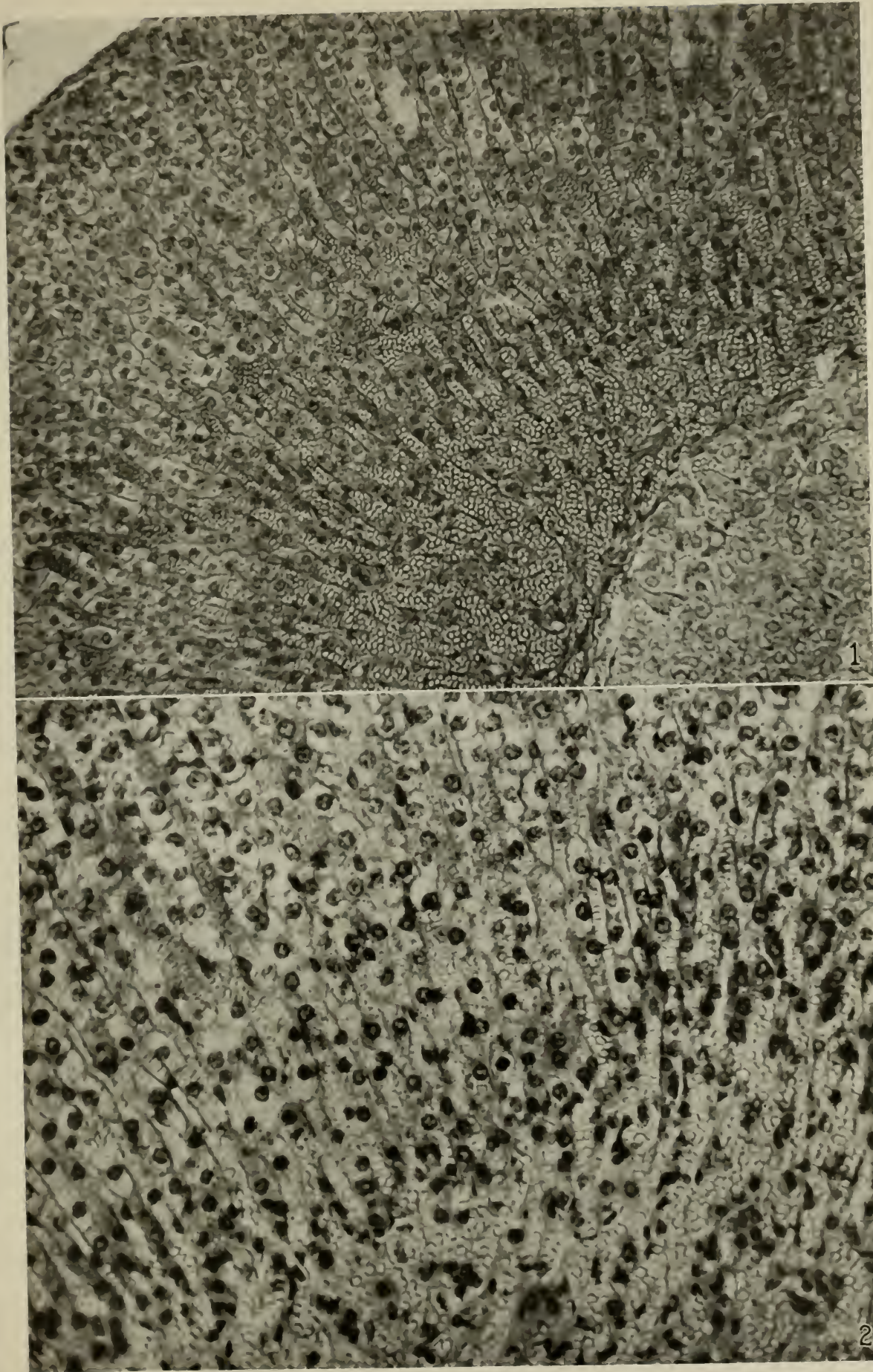
FIG. 1. Suprarenal gland of mouse 7 days after an exposure to low frequency x-rays, showing dilatation and engorgement of capillaries.

FIG. 2. The same as Fig. 1; higher power view.

PLATE 33.

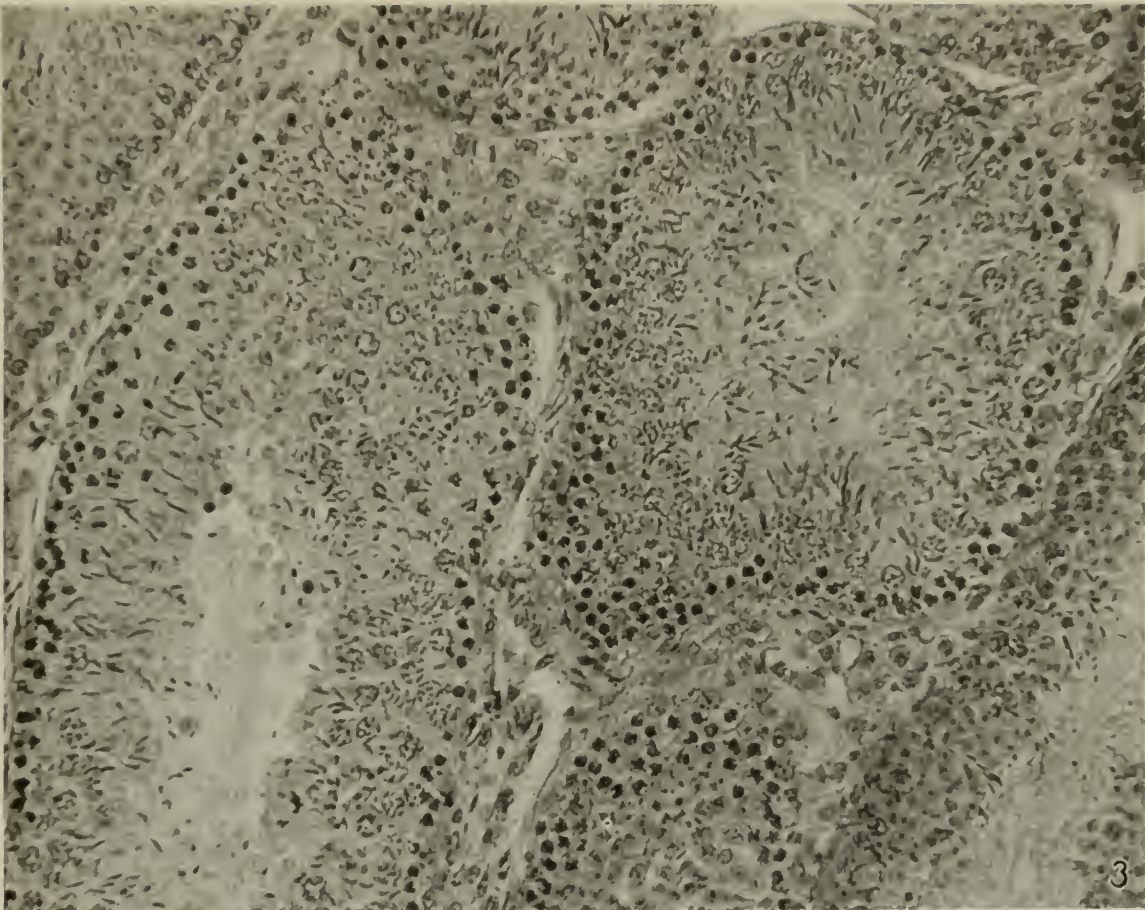
FIG. 3. Testis of mouse 24 hours after an exposure to low frequency x-rays. Spermatocytes in the periphery of follicles in the so called syncopic stage. In the large follicle to the left are seen several examples of the first spermatocytic division.

FIG. 4. Ovary of mouse 24 hours after an exposure to low frequency x-rays.

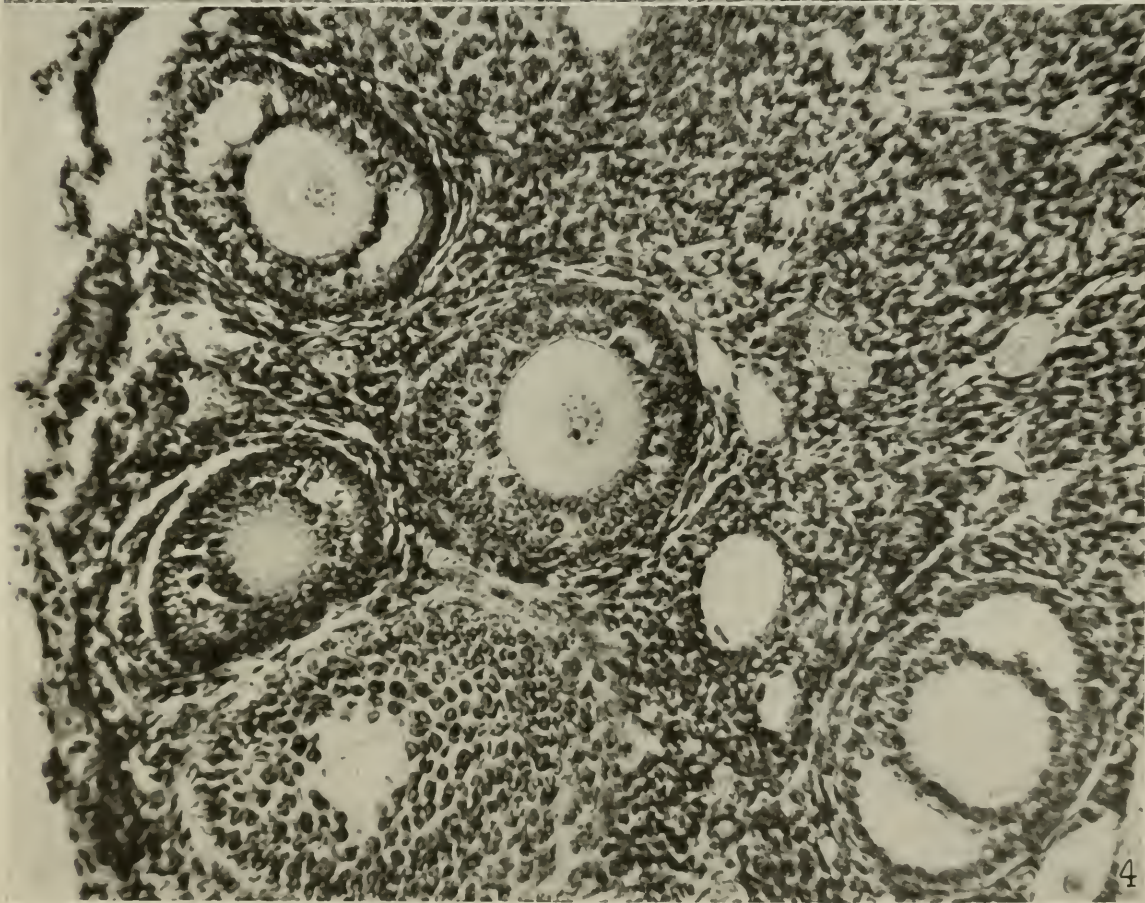


(Nakahara and Murphy: Studies on x-ray effects. X.)

486



3



4

(Nakahara and Murphy: Studies on x-ray effects. X.)



STUDIES ON X-RAY EFFECTS.

XI. THE FATE OF CANCER GRAFTS IMPLANTED IN SUBCUTANEOUS TISSUE PREVIOUSLY EXPOSED TO X-RAYS.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 22, 1921.)

It has been shown in this laboratory¹ that an erythema dose of x-rays produces in the skin layers of mice a reaction characterized by lymphoid infiltration coincident with a local increase in resistance to transplanted cancer. Moreover, it was found that while the x-rayed areas are refractory to subsequent intracutaneous inoculation of cancer, subcutaneous inoculations beneath the x-rayed areas result in the same number of growths as in the normal areas. These experiments were offered as a probable explanation of the fact that many skin cancers in man are readily influenced by x-rays while identical cancers in the superficial lymph nodes are controlled with great difficulty, if at all, by the treatment.

The exposure of open wounds to x-rays at the time of operation for the removal of cancer in man has been frequently advocated, particularly in cancer of the breast² for the purpose of destroying any remaining cancerous tissue. While the results are stated to have been satisfactory, it is difficult to judge the value of the method in the treatment of human cancer in which it is impossible to provide a suitable number of controls.

Hill, Morton, and Witherbee have shown that mouse cancer cells are not killed by x-rays *in vitro* in doses much greater than those tolerated by the skin.³ Yet as stated above, a much smaller dose will render the skin resistant to implants of a strain of this same cancer.

¹ Murphy, Jas. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 299.

² Pfahler, G. E., in Deaver and McFarland, *The breast, its anomalies, its diseases and their treatment*, Philadelphia, 1917, 651.

³ Hill, E., Morton, J. J., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 89.

Hence it became desirable to determine whether or not direct exposure of the deeper tissues renders them refractory to implanted cancer.

Tumor Inoculation after Exposure of the Subcutaneous Tissue to X-Rays.

Normal white mice were etherized, shaved over the abdomen, and under aseptic conditions a rectangular skin flap was made beginning at the midline and extending about 1.5 cm. laterally across the abdomen. The incision was made so as to leave the skin attached at the distal end and the flap was separated from the underlying structures so as to include all of the subcutaneous tissue down to the muscle. The under side of the flap and the exposed muscle, after being covered with gauze wet with salt solution, were given directly a dose of x-rays governed by the following factors: 3 inch spark-gap, 10 milliamperes, 6 inch distance, $2\frac{1}{2}$ minutes. With the exception of this area the animal's body was protected by sheet lead. Immediately after the treatment a cancer graft was introduced into the loose connective tissue of the under side of the flap and the skin sutured back into place. As a control, another series of animals was treated in precisely the same fashion except that no x-rays were given.

In practically all of the animals of both series the wounds healed within 5 or 6 days by primary intention, with no detectable difference between the x-rayed and control animals. Weekly examinations were made to determine the fate of the cancer grafts and later verified by autopsy.

The results of four such experiments are given in Table I.

TABLE I.

Experiment No.	Resistant x-rayed mice.	Resistant control mice.
1	66.6 per cent (15 mice).	23.0 per cent (13 mice).
2	68.4 " " (19 ").	0.0 " " (6 ").
3	73.3 " " (15 ").	29.4 " " (17 ").
4	50.0 " " (10 ").	0.0 " " (10 ").
Average...	66.1 per cent (59 mice).	17.4 per cent (46 mice).

It is apparent from these experiments that an erythema dose of x-rays given directly to the subcutaneous tissue brings about some change which renders this tissue decidedly less suitable as a soil for the growth of implanted cancer (Text-fig. 1). Another point of

Subcutaneous tissue x-rayed Tumor inoculated immediately after				Controls		
1	—	—	—	• ?	—	—
2	—	—	—	+ ?	—	—
3	—	—	—	— ?	—	—
4	— ?	—	—	— ?	—	—
5	—	—	—	+ ?	—	—
6	+ ?	+ ?	—	•	•	•
7	—	—	—	•	•	•
8	—	—	—	+ ?	•	•
9	—	—	—	•	•	•
10	+	—	—	+ ?	•	•
11	—	—	—	•	•	•
12	— ?	•	•	•	•	•
13	—	•	Died	— ?	•	•
14	+ ?	•	•	•	•	•
15	—	•	•	•	•	•
16				•	•	•
17				•	•	•
Weeks 1	2	3		1	2	3

TEXT-FIG. 1. Results of inoculation of tumor into subcutaneous tissue previously exposed directly to x-rays, compared with a like inoculation in normal mice.

interest is that the cancer grafts which took in the x-rayed animals showed a tendency to grow inward toward the abdominal cavity with a flat inactive base on the side near the skin. Some of these did not produce even a slight elevation of the overlying skin and were only detected at autopsy.

To serve as a control for the above experiments and in order to confirm the finding that the effect of a local erythema dose of x-rays applied to the skin does not extend to the underlying subcutaneous tissue, the following experiment was carried out.

Subcutaneous Inoculation of Tumor after Exposure of the Skin to X-Rays.

Ten normal white mice were given a dose of x-rays over the left lower quadrant of the abdomen, the dose being governed by the same factors as those used in the preceding experiment. Immediately following the exposure, a skin flap was made in the x-rayed area and a cancer graft (Bashford No. 63) was introduced into the loose areolar tissue of the under side of the flap and the wound closed with sutures. The tumor grew in all of these animals, from which it may be concluded that an erythema dose of x-rays given to the intact skin does not increase the resistance of the underlying subcutaneous tissue.

Tumor Inoculation in a Protected Area after a Local Exposure of the Subcutaneous Tissue to X-Rays.

In order to determine whether the exposure to x-rays of a small area of subcutaneous tissue affects the general resistance to cancer grafts, a series of thirteen mice was operated upon and after the skin flap was made on the left side of the abdomen they were x-rayed over the open wound and then the flap was sutured back into place, Cancer grafts inoculated immediately afterwards in the right side resulted in tumors in 76.9 per cent of the animals, or in about the proportion observed in normal control mice.

Histological Changes after Direct Exposure of the Subcutaneous Tissue to X-Rays.

Two series of twelve mice each were shaved, and under ether flaps of skin and subcutaneous tissue were made over the left lower abdominal region. One series was x-rayed with an erythema dose directly on the under side of the skin flap and on the denuded surface of the abdominal muscle, the remaining parts of the animal being protected by sheet lead. The other series was operated on in the

same manner but not x-rayed. The wounds in both were sutured with great care as to the approximation of the skin edges. The animals were killed in groups of two, 24 hours, 3, 5, 7, 9, and 14 days after operation for examination.

Up to the 5th day the process of repair formed such a prominent part of the picture that it was impossible to detect any difference in the extent and character of the cellular infiltration from histological study. The 7 and 9 day preparations, however, in which the process of repair was in the last stages, showed distinctly that while in the animals not x-rayed only a layer of new connective tissue between the subcutaneous and muscle layers was slightly infiltrated with round cells, in the x-rayed animals large numbers of lymphocytes occurred, chiefly in the loose connective tissue, and in about half of the animals examined these cells had infiltrated the thickness of the muscle and formed a heavy layer between the muscle and the parietal peritoneum. At the end of 2 weeks this lymphocytic infiltration, although still evident, had subsided somewhat.

Two other groups of mice were operated on in the same manner as in the preceding experiment, and one of the groups was given a dose of x-rays over the exposed subcutaneous tissue and muscle. Before the skin flap was sutured back into place each animal received a cancer graft into the connective tissue underlying the flap. The microscopic appearances of the sections of tissue taken at intervals from the animals were so complicated, through operation, x-ray treatment, inoculation of tumor, natural differences in susceptibility, and in some cases, mild infections, that no conclusions in regard to the cellular reactions could be drawn.

DISCUSSION.

The observations reported in this paper bring out the fact that x-rays can be made to induce a local change in the subcutaneous tissue similar to that which this agent will induce in the skin. This change, in both instances, renders the locality resistant to the growth of implanted cancer cells, but does not affect the general resistance of the animal. This is an additional point to be taken into consideration in determining the method of treatment and the interpretation of clinical results following the use of x-rays as a therapeutic agent. The

clinician has rarely taken into account other possibilities than the direct destruction of the cancer cells.

X-rays under certain conditions materially increase the general resistance of the body to cancer, an observation made in this laboratory,⁴ and later confirmed and extended by Russ, Mottram, and their coworkers.⁵ On the other hand, excessive doses of x-rays are capable of lowering both natural and induced resistance to cancer.⁶ The amount of this agent required to kill mouse cancer cells is many times that which can be tolerated by the skin, yet as shown above a mild erythema dose is sufficient to render the skin and under proper conditions the subcutaneous tissue antagonistic to the growth of implanted cancer. It is undetermined which of these various qualities of x-rays are responsible for the successes and failures in the treatment of human cancer. It would seem of prime importance to estimate the relative value of these effects, for it is not beyond the possible that a method of treatment could be devised which would make use of the favorable and eliminate the unfavorable action of this agent.

SUMMARY.

An erythema dose of x-rays given direct to the exposed subcutaneous tissue and muscle greatly diminishes the susceptibility of the exposed area to transplanted cancer. The same dose given over the intact skin does not affect the resisting power of the underlying subcutaneous tissue.

Histological examination shows that a few days after the exposure of the subcutaneous tissue there is a lymphoid infiltration of this tissue, which infiltration sometimes includes the muscle layers as well.

⁴ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35. Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

⁵ Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692. Russ, S., Chambers, H., and Scott, G. M., *Proc. Roy. Soc. London, Series B*, 1921, xcii, 125.

⁶ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204. Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1. Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917, xc, 1.

STUDIES ON LYMPHOID ACTIVITY.

VI. IMMUNITY TO TRANSPLANTED CANCER INDUCED BY INJECTION OF OLIVE OIL.

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PLATES 34 TO 36.

(Received for publication, October 31, 1921.)

It has been frequently suggested that the lymphoid cells are in some way concerned with the absorption and digestion of fats and lipoids. Recently a number of investigators have reported on the cellular changes following injections of these substances. Ramond¹ found that olive oil injected intraperitoneally is gradually absorbed by white cells of the lymphoid variety, and Clark² noted that the subcutaneous injection of olive oil exerts what he considers to be a chemotactic influence on the lymphatic endothelium and lymphocytes. Bergel³ confirmed and extended these observations by finding that the cellular exudate after an intrapleural or intraperitoneal injection in animals of fatty oil or oil emulsion of lecithin is almost entirely made up of the lymphoid type of cell.

It is well known that the local reaction following an injection of homologous living tissue in mice consists mainly of a lymphoid cell outpouring⁴ similar to that described above. Murphy and Nakahara⁵ observed that this local reaction is accompanied by evidences of increased proliferative activity among the lymphoblastic cells of the spleen and lymph nodes. It may be stated in passing that mice thus injected with homologous tissue become highly resistant to

¹ Ramond, M. F., *Compt. rend. Soc. biol.*, 1904, lvi, pt. 2, 95.

² Clark, E. R., and Clark, E. L., *Am. J. Anat.*, 1917, xxi, 421.

³ Bergel, S., *Berl. klin. Woch.*, 1919, lvi, 915; *Z. exp. Path. u. Therap.*, 1920, xxi, 216; *Ergebn. inn. Med.*, 1921, xx, 36.

⁴ Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

⁵ Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

transplanted cancer.⁶ A like stimulation of the proliferative activity of the lymphoid cells may be induced by certain physical agents,⁷ with resultant increased resistance to cancer transplants.⁸

In view of these observations it was regarded as of interest to determine whether or not the local reaction to oil is accompanied by a general lymphoid stimulation and, if so, the effect on the resistance to cancer inoculation in mice.

General Lymphoid Response to Injections of Olive Oil.

Commercial olive oil,⁹ described as the first expression, was used in the following experiments. The injections were made intraperitoneally, followed by a histological study of the general condition of the lymphoid organs, with special attention to the number of mitotic figures present as this had been shown to be a fair index of the degree of stimulation.¹⁰

Experiment 1.—Twenty-five normal white mice of about the same age and size were divided into five groups of five mice each. The mice of Group A received an intraperitoneal injection of 0.1 cc. of the oil, Group B received 0.2 cc., Group C, 0.3 cc., Group D, 0.5 cc., and Group E, 0.7 cc. One animal from each group was killed for histological study 24 hours, 48 hours, 3 days, 4 days, and 5 days after the injection.

Group A.—Mice of this group received each 0.1 cc. of the oil. No unusual features were found in lymphoid organs of any of the mice, excepting one that was killed 4 days after the injection, and the germ centers of the lymphoid tissue of this mouse showed a marked increase of mitotic figures.

Group B.—Mice of this group received each 0.2 cc. of the oil. The mouse killed 24 hours after the injection showed no unusual condition, but four others killed at 48 hours to 5 days did show, particularly in germ centers (Fig. 1), a definite increase in mitosis.

⁶ Bashford, E. F., *Brit. Med. J.*, 1906, ii, 209.

⁷ Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

⁸ Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

⁹ It was estimated that this product contained about 30 per cent of cottonseed oil. This was determined by the density of the brown color following the addition of nitric acid, using as a standard known dilutions of the cottonseed oil.

¹⁰ Nakahara, W., and Murphy, Jas. B., *Anat. Rec.*, 1921, xxii, 107.

Group C.—Mice of this group received each 0.3 cc. of oil. An appreciable but less marked increase in the number of mitotic figures was present 48 hours to 5 days after the injection.

Group D.—Mice of this group each received 0.5 cc. of oil. Judged by the number of mitotic figures, proliferative activity was retarded in the 24 and 48 hour specimens and about normal in the remaining animals.

Group E.—Mice of this group each received 0.7 cc. of the oil. In mice killed at the 24 and 48 hour periods, the lymphoid organs, especially the spleens, were much reduced in size. These small lymphoid organs showed an almost complete suppression of mitosis, reduction in the amount of lymphoid tissue, and, in the splenic pulp, vacuoles of various sizes. At later periods, while the size of lymphoid organs was still small, the rate of mitosis was approximately normal.

The preceding experiment indicates that the most pronounced reaction in the lymphoid organs followed the intraperitoneal injection of a dose of 0.2 cc. of the oil.

Experiment 2.—Twelve normal white mice were injected intraperitoneally with 0.2 cc. of olive oil as in Group B of the preceding experiment. The mice were killed in groups of three each at 48 hour, and 4, 7, and 10 day intervals.

Of the three mice killed 48 hours after the injection, one showed unmistakable increase in the number of mitotic figures in the lymphoid germ centers, another a less striking but distinct increase, and a third mouse no appreciable increase.

At 4 and 7 day periods, all the mice showed a greatly increased number of mitotic figures. In one mouse killed at the 4 day period the mitosis was especially exaggerated, and was present not only in, but outside the germ centers.

At the 10 day period, mitotic figures were still abundant in two mice and there were a few in the third.

As a control to the above experiment, a number of normal mice were injected intraperitoneally with 0.2 cc. of liquid petrolatum without leading to an increase of the mitosis.

Other Organs.—The livers of a few of the mice injected with oil had many intracellular vacuoles suggestive of fatty inclusions. Also occasionally there were found a marked dilatation of the capillaries and sinus-like spaces of the suprarenal gland and an increase in the number of mitotic figures in the cortical cells (lymphocytes) of the thymus. These findings were of irregular occurrence and, therefore, should not be classed as typical changes induced by the oil injection. No special alterations were noted in the thyroid glands, kidneys, or bone marrow.

Cytology of the Peritoneal Exudate.—Smears were taken of peritoneal fluid at autopsy in Experiments 1 and 2. As already pointed out by Bergel,³ all the smears showed numerous cells of lymphoid group, including typical large and small lymphocytes, so called transitional cells, plasma cells, large cells resembling macrophages, and true endothelial cells. Morphologically, there is a complete series of intergradations from the typical small lymphocyte to the large macrophage-like cell, suggesting that they all belong to a single biologic group, a point which has been emphasized by Bergel.³ At an early stage (24 hours after the oil injection), there is a considerable number of polymorphonuclear cells, particularly neutrophils and eosinophils, in addition to lymphoid cells, but the number of these granular cells soon falls off. At the 48 hour period, the lymphoid reaction is apparently at its height, while at this time the polymorphonuclear reaction has about subsided (Fig. 2). The local reaction occurs regardless of the amount of oil injected and lasts as long as the oil remains in the peritoneal cavity. When 0.2 cc. of olive oil was injected the reaction tended to subside within 10 days, or hand in hand with the gradual absorption of the oil.

The above experiments indicate that lymphoid tissue as a whole responds definitely to an intraperitoneal injection of olive oil, which, if given in the optimum quantity, brings about a marked stimulation of the proliferative activity of this tissue. Studies on the peritoneal exudates, moreover, confirmed the results reported by Ramond,¹ Clark,² and Bergel,³ regarding the local cellular manifestations about the injected fatty and lipoidal substances.

The relation between the local lymphoid reaction and the stimulation of the germ centers cannot be determined directly. However, in view of the lipolytic function of lymphoid cells,¹¹ it does not seem improbable that the local lymphoid response to the injected oil is an expression of the attempt of the body to dispose of the injected material. If so, it is conceivable that an optimum grade of activity on the part of lymphoid cells may lead to the general lymphoid stimulation.

¹¹ Bergel, S., *Münch. med. Woch.*, 1909, lvi, 64. Fiessinger, N., and Marie, P.-L., *Compt. rend. Soc. biol.*, 1909, lxxvii, pt. 2, 177.

Cancer Inoculation Experiments.

The production by injection of olive oil of lymphoid stimulation, essentially similar to the condition of induced potential immunity to transplanted cancers, suggested the possibility of rendering animals resistant by the same method. Experiments were accordingly undertaken to determine this point.

The dose of olive oil was given in a single intraperitoneal injection. Cancer inoculation was made subcutaneously in the left groin, and the rate of growth of tumors was charted thereafter at weekly intervals for 3 weeks. The strain of cancer used was Bashford Adenocarcinoma No. 63, and all the mice were young adults of white variety from the same stock.

The Degree of Resistance in Relation to the Amount of Olive Oil Injected.

Experiment 3.—In order to determine the optimum dose of olive oil, this substance was injected into mice in different quantities, ranging from 0.1 to 0.7 cc. Cancer inoculation was made in every case 10 days after the injection. The results are summarized in Table I.

TABLE I.
Experiment 3.

Amount of olive oil.	Treated mice.		Controls.	
	Resistance.	No. of mice.	Resistance.	No. of mice.
cc.	per cent		per cent	
0.1	20.5	19	0.0	10
0.2	40.0	18	0.0	19
0.3	25.0	20	5.5	21
0.5	6.1	23	11.1	9
0.7	0.0	9	0.0	9

Since the number of mice in each group is small, slight differences in the percentage of resistance in the several groups should not be considered as significant. However, the fact that emerges is that by injecting 0.2 cc. of olive oil 10 days before giving a cancer inoculation, mice are rendered more resistant to the inoculated cancer than they

normally are. This same point was brought out in three additional experiments, the results of which are shown in Table II.¹²

TABLE II.
Experiments 4 to 6.

Experiment No.	Mice injected with olive oil 10 days before cancer inoculation.		Controls.	
	Resistance.	No. of mice.	Resistance.	No. of mice.
	<i>per cent</i>		<i>per cent</i>	
4	43.7	16	11.1	9
5	52.6	19	10.0	10
6 (Text-fig. 1).	41.0	20	10.5	19

The Degree of Resistance in Relation to the Time of Cancer Inoculation.

Experiment 7.—In the preceding experiment the cancer inoculations were made 10 days after the oil injection. In order to ascertain the period at which the maximum degree of resistance is manifested, the inoculations in the following experiment were made at various intervals after an injection of 0.2 cc. of the oil. The results are shown in Table III.

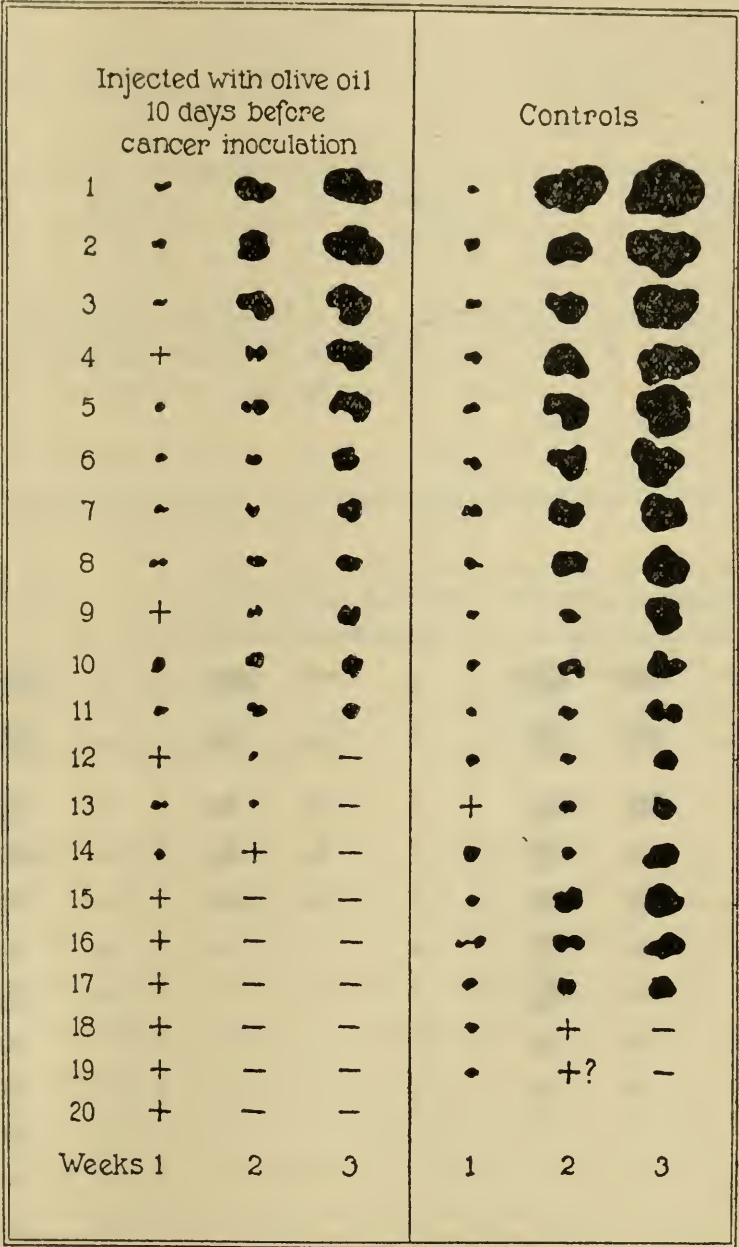
TABLE III.
Experiment 7.

Intervals between oil injection and cancer inoculation.	Resistance.	No. of mice.
<i>days</i>	<i>per cent</i>	
5	20.0	10
10	44.4	9
15	30.0	10
25	10.0	10
Control.	0.0	12

In all the former types of induced resistance to transplanted cancer so far studied, there is a period following the treatment during which there is slight, if any, evidence of resistance. This is not only true as

¹² The amount of olive oil should be slightly changed according to the size of the mouse. For a large mouse, weighing over 25 gm., as much as 0.3 cc. can be given.

regards the injection of homologous living tissue,¹³ but also equally after exposure to intense dry heat and after small doses of x-rays.¹⁴



TEXT-FIG. 1. Experiment 6. The rate of growth of Bashford Adenocarcinoma No. 63 in mice injected with 0.2 cc. of olive oil 10 days before inoculation, contrasted with the rate of growth in untreated mice.

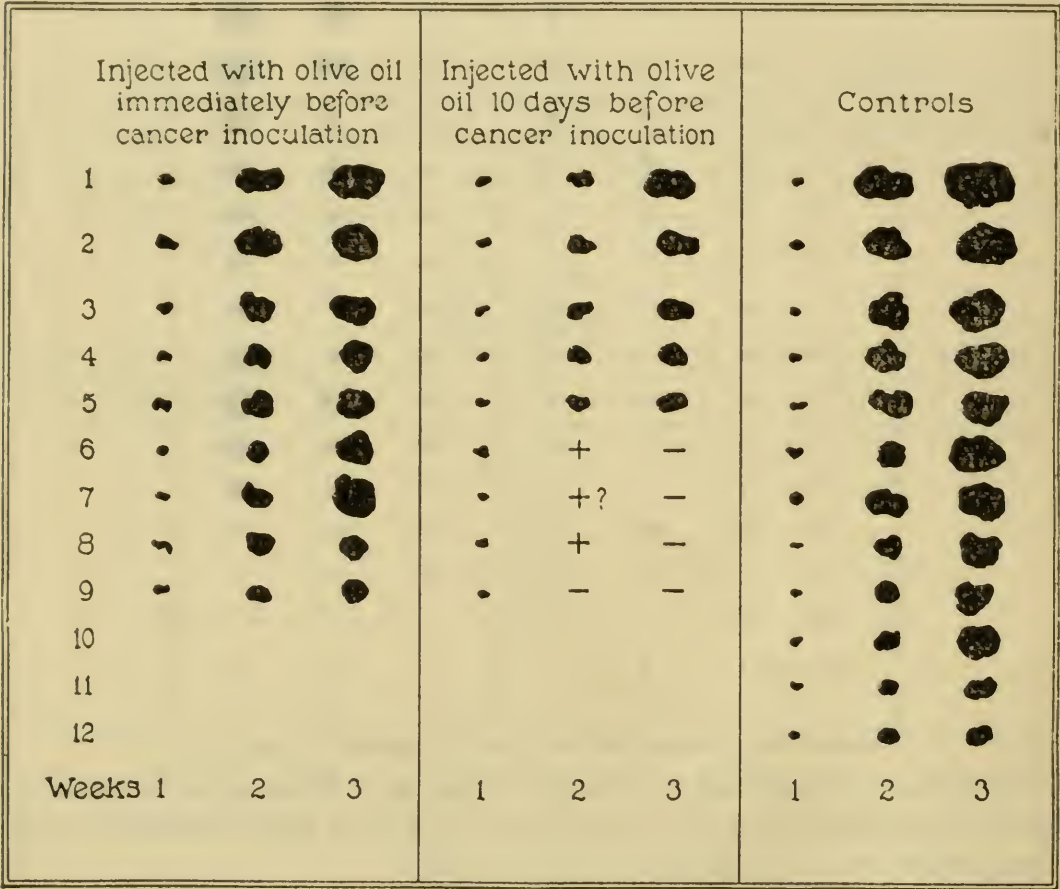
¹³ Bashford, E. F., Murray, J. A., and Cramer, W., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 180. Woglom, W. H., *J. Exp. Med.*, 1912, xvi, 629.

¹⁴ Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35. Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

TABLE IV.
Experiments 8 to 10.

Experiment No.	Resistance.		
	Group A.*	Group B.	Group C.
8 (Text-fig. 2).	44.4 per cent (9 mice).	0.0 per cent (9 mice).	0.0 per cent (12 mice).
9	30.0 " " (10 ").	10.0 " " (10 ").	0.0 " " (10 ").
10	50.0 " " (8 ").	10.0 " " (10 ").	12.5 " " (8 ").

* Group A was made up of mice injected with the oil 10 days before the cancer inoculation. Group B mice were injected with the oil immediately before the inoculation. Group C comprised the control mice receiving no oil and inoculated with the same tumor.



TEXT-FIG. 2. Experiment 8. Effect of 0.2 cc. of olive oil on the rate of growth of Bashford Adenocarcinoma No. 63, when administered 10 days and immediately before inoculation.

That a similar state arises after an injection of olive oil is shown by the above experiment. A more complete test of this point is given in Table IV.

Other Oils.—Several other oils of different chemical constitution have been tested; namely, cod liver oil, cocoanut oil, sperm oil, and liquid petrolatum (Nujol). Cancer inoculations made 10 days after injection of 0.2 cc. of these several oils induced no appreciable resistance. The tests do not, however, exclude the possibility of a suitable dosage of these oils inducing a result comparable to that given by olive oil.

Histological Changes Accompanying the Resistance Induced by Olive Oil.

The following experiments were carried out in order to supply material for a histological study of the nature of the reaction accompanying the resistant state induced by olive oil.

Experiment 11.—Ten normal white mice were given an intraperitoneal injection of 0.2 cc. of olive oil each. 10 days later they were inoculated with fragments of a Bashford Adenocarcinoma No. 63, subcutaneously in the left groin. The mice were then killed in pairs 24 hours, 48 hours, 3 days, 4 days, and 5 days after the inoculation and the grafts and the lymphoid organs were removed for histological study.

Local Cellular Infiltration.—The occurrence of a characteristic exudate around the cancer grafts in resistant animals has long been known.¹⁵ This local reaction, in which the cells of lymphoid variety take a prominent part, subsides rapidly after the necrosis of the grafts has become complete. On this account the grafts in the present experiment were removed at early periods.

Specimens taken 24 and 48 hours after inoculation showed various types of wandering cells, especially polymorphonuclear leucocytes and fibroblasts, collecting in a great number around the graft. At the 3 day period, however, much of the polymorphonuclear reaction had subsided and there was a marked infiltration of lymphocytes, plasma cells, and fibroblasts (Figs. 3 and 4) closely resembling the local re-

¹⁵ Burgess, A. M., *J. Med. Research*, 1909, xxi, 575. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1912, xv, 270. Tyzzer, E. E., *J. Med. Research*, 1915, xxxii, 201.

action known to occur in cancer-resistant animals. Cell infiltration similar to the latter but in varying amounts was encountered in all the specimens taken at 4 and 5 day periods.

Stimulation of Lymphoid Tissue.—Animals resistant to cancer inoculation tend to develop on inoculation lymphoid hyperplasia.^{16,17} Murphy and Nakahara^{5,17} have shown that this phenomenon, which is characterized by a marked increase in the number of mitotic figures in germ centers of lymphoid tissue, occurs very soon after cancer grafting in potentially resistant animals.

Spleens and lymph nodes taken as early as 24 hours after cancer inoculation in the above experiment showed that the number of mitotic figures in lymphoid tissue was greater than is seen in normal animals. At the 48 hour and 3 day periods the reaction appears to reach its height and at the latter periods mitotic figures were found in great numbers in the germ centers of the spleen (Fig. 5) and lymph nodes, and often in considerable numbers even in the lymph cord of the node (Fig. 6). It should be stated that one animal each of the 4 and 5 day period failed to show any increase of mitotic figures, an irregularity without significance.

Blood Lymphocytosis.—Murphy and his associates have shown that a marked increase in the number of circulating lymphocytes accompanies the state of resistance to transplanted cancer.¹⁸ In order to ascertain whether or not such a lymphoid crisis occurs after cancer inoculation in the mice treated with olive oil, white cell counts were made of a number of such mice.

Experiment 12 (Text-Fig. 3).—Nineteen normal white mice were injected intraperitoneally with 0.2 cc. of olive oil and cancer inoculation was made in all of the mice 10 days afterward. Ten of the mice proved to be resistant and nine susceptible to the inoculation.

The average number of lymphocytes per c.mm. of blood in the resistant mice, 1 day before the oil injection, was about 4,800 and of polymorphonuclear leucocytes about 4,700. 3 days after cancer inoculation the lymphocytes increased

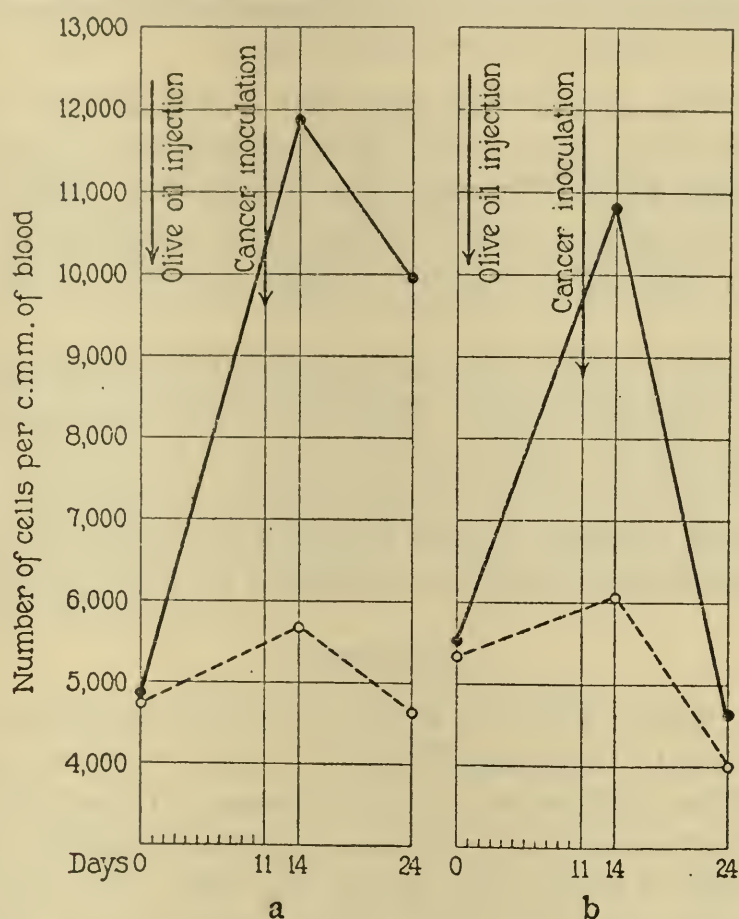
¹⁶ Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917-18, xc, 1.

¹⁷ Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 327.

¹⁸ Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 433.

to about 11,800, while the polymorphonuclear cells were only slightly increased, being about 5,600. 2 weeks after cancer inoculation the lymphocyte count was still high, being about 10,000, while the polymorphonuclear leucocytes had returned to the initial level (about 4,600).

In cancer-susceptible mice, 1 day before the oil injection, the average number of lymphocytes and polymorphonuclear leucocytes was about 5,500 and 5,400



TEXT-FIG. 3. Experiment 12. Composite curves of white cell counts on mice injected with 0.2 cc. of olive oil and inoculated with Bashford Adenocarcinoma No. 63, 10 days later. (a) Composite curves from ten mice proved to be immune; (b) composite curves from nine mice proved to be susceptible. — Lymphocytes. Polymorphonuclears.

respectively. The counts were quite high 3 days after the inoculation, the lymphocytes being about 10,800 and the polymorphonuclear leucocytes about 6,000. At the end of the 2nd week after the cancer inoculation, all the cells were much reduced, the lymphocytes being about 4,600 and polymorphonuclear leucocytes about 4,000.

Experiment 13.—An experiment similar to the preceding one was carried out with ten mice, five of which were resistant and five susceptible.

In the resistant mice the average number of lymphocytes per c.mm. of blood 1 day before the oil injection was about 4,000; polymorphonuclear leucocytes about 4,400. 2 weeks after the cancer inoculation the lymphocytes were a little over 8,000 while the polymorphonuclear leucocytes had decreased to about 3,400.

In the susceptible mice, 1 day before the oil injection the lymphocytes were about 3,600 and the polymorphonuclear leucocytes about 4,000. 2 weeks after cancer inoculation the lymphocytes were unchanged and the polymorphonuclear leucocytes had perceptibly increased (about 5,700).

The preceding experiments show that a characteristic lymphoid crisis occurs in the blood during the establishment of resistance to cancer grafting induced through olive oil injection. Experiment 12 suggests that even in mice that proved to be susceptible there is apparently an inadequate reaction, which, however, is not of long duration.

It should also be stated that the injection of olive oil alone does not bring about a lymphocytosis. White cell counts were made on ten normal mice injected with 0.2 cc. of olive oil. In certain of the mice there was an increase and in others a decrease in number of circulating lymphocytes during the first 10 days after the oil injection, but the changes were too slight and too irregular to be of importance.

DISCUSSION.

The early work of Bashford and his coworkers⁶ established the fact that resistance to transplanted cancer in mice could be induced by the inoculation of homologous living tissues. Later Murphy and his associates¹⁴ showed that resistance could be induced by the use of suitable doses of x-rays and intense dry heat. The experiments reported in this paper demonstrate that resistance may be induced by still another means; namely, by the intraperitoneal injection of olive oil. Thus it may be said that resistance to transplanted cancer can be induced by three classes of agents—homologous tissue, a biological agent; x-rays and heat, physical agents; and olive oil, a chemical agent.

There is little direct indication concerning the nature of the common factors responsible for the resistant state induced by these various agents but the manifestations associated with phenomena of resistance are the same regardless of the means used to induce this state. These associated manifestations are, a latent period after the treatment, during which time there is no evidence of resistance,

a local cellular reaction about the inoculated cancer graft, an increase in the number of circulating lymphocytes, and a marked increase in the proliferative activity in the lymphoid organs. The indirect evidence associating the lymphoid cell with the mechanism of resistance to cancer is so strong as to leave little doubt that this cell has an important, if not the most important rôle in bringing about the resistant state.

SUMMARY.

The experiments reported in this paper show that it is possible to render mice resistant to transplanted cancer by injections of a suitable quantity of olive oil. In the course of the development of the resistance a definite period of latency is detectable following the oil injection, and the maximum degree of resistance appears at about the 10th day. This state of resistance, as has been determined by histological studies, is preceded by a proliferation of the cells of the lymphoid germ centers and, after the cancer inoculation, is associated with a lymphoid infiltration about the grafts, as well as by a second stimulation of the lymphoid germ centers and an increase in the number of the circulating lymphocytes.

EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Germ center of spleen 4 days after an intraperitoneal injection of 0.2 cc. of olive oil. *M*, mitotic figure.

FIG. 2. Peritoneal exudate 48 hours after an intraperitoneal injection of 0.2 cc. of olive oil.

PLATE 35.

FIG. 3. 48 hour old cancer grafts and surrounding connective tissue in mouse injected with 0.2 cc. of olive oil 10 days previous to cancer inoculation. Note the extensive cellular infiltration around the graft.

FIG. 4. High power view of an area of infiltration in the above specimen, showing the types of cells participating in the infiltration.

PLATE 36.

FIG. 5. Germ center of spleen of mouse injected with 0.2 cc. of olive oil and inoculated with cancer 10 days later. 3 days after cancer inoculation. *M*, mitotic figure.

FIG. 6. Medulla of lymph node of mouse treated similarly to that of Fig. 5. 3 days after cancer inoculation. *M*, mitotic figure.

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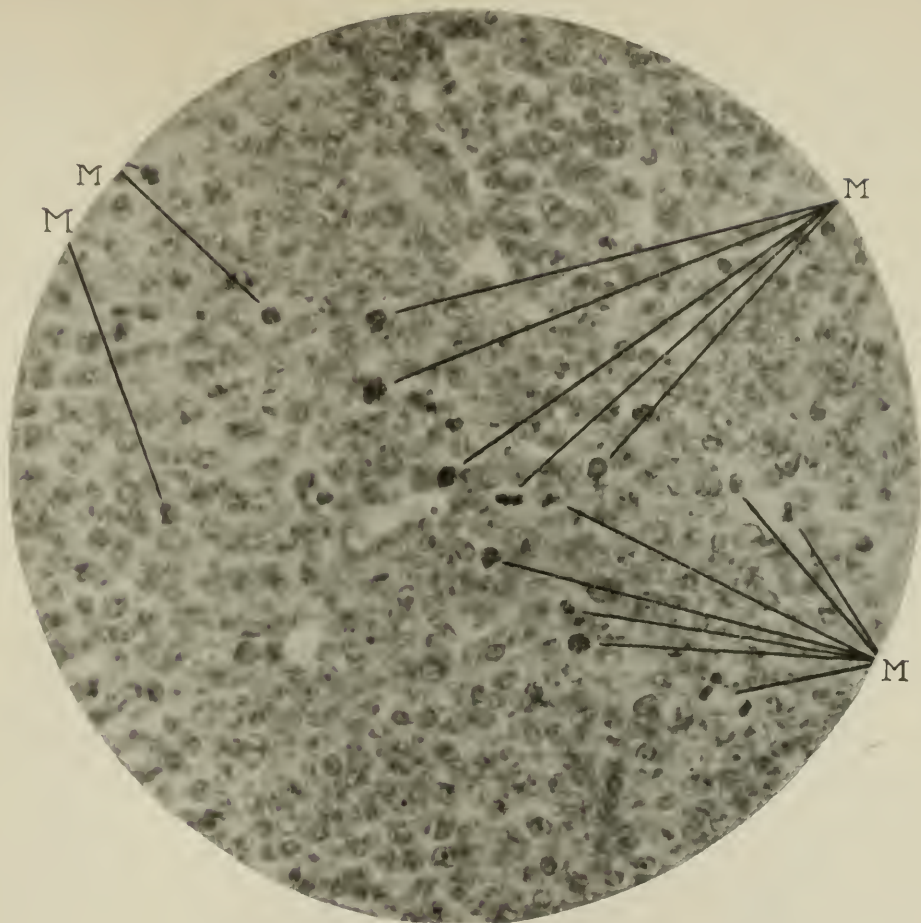


FIG. 1.

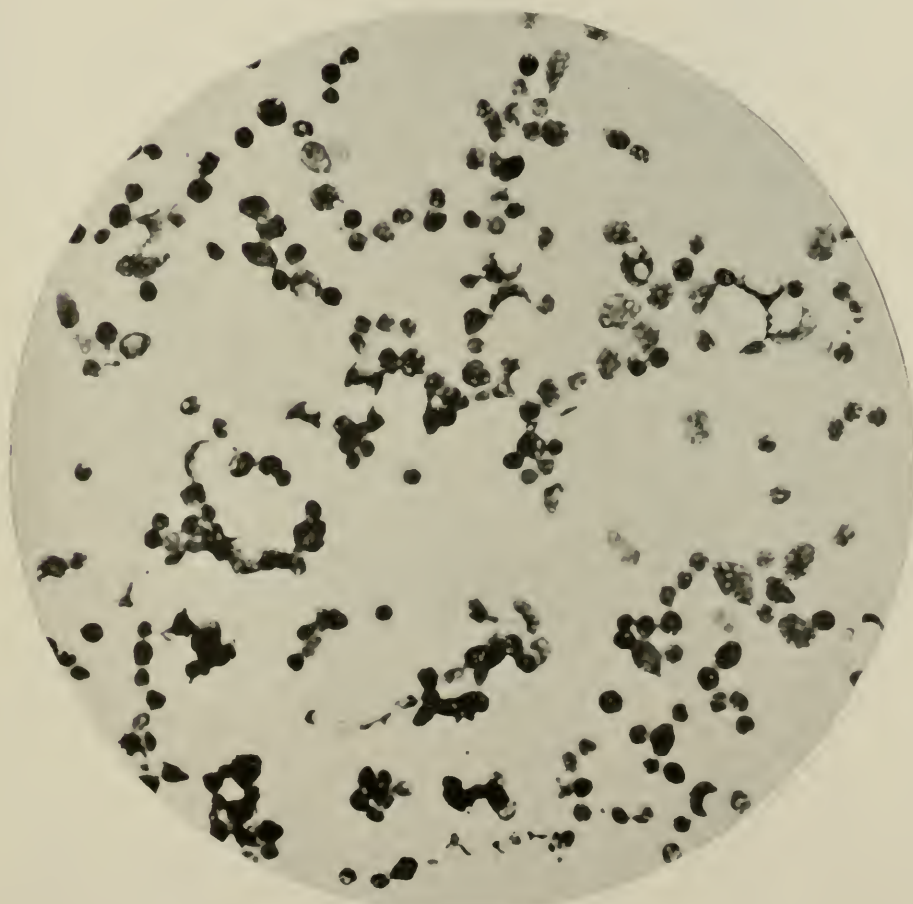


FIG. 2.

(Nakahara: Lymphoid activity. VI.)



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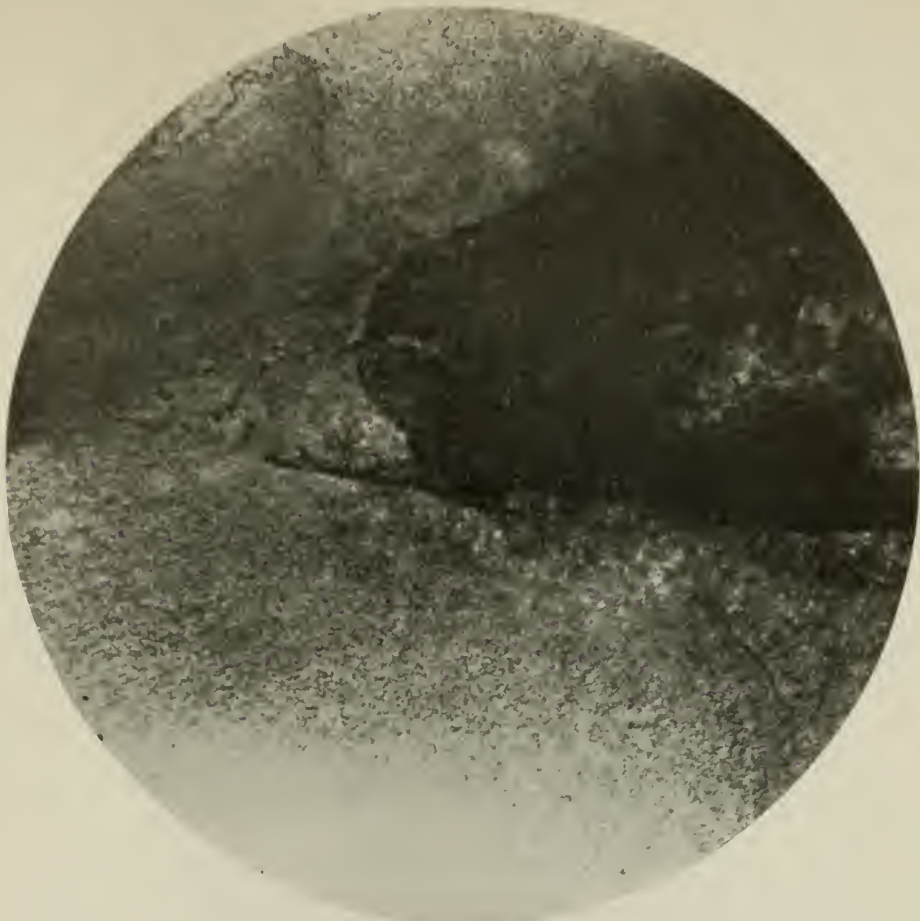


FIG. 3.

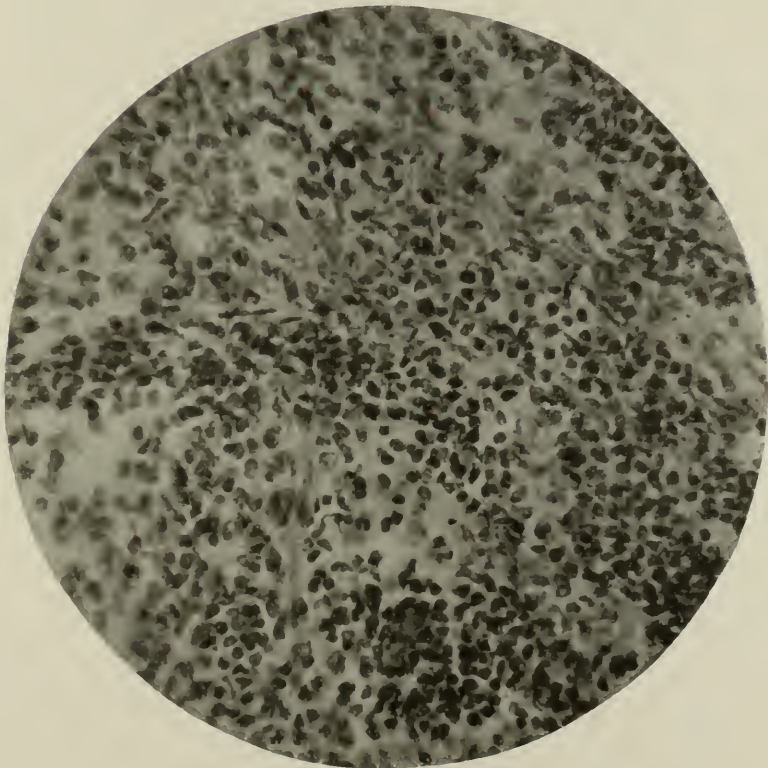


FIG. 4.

(Nakahara: Lymphoid activity. VI.)

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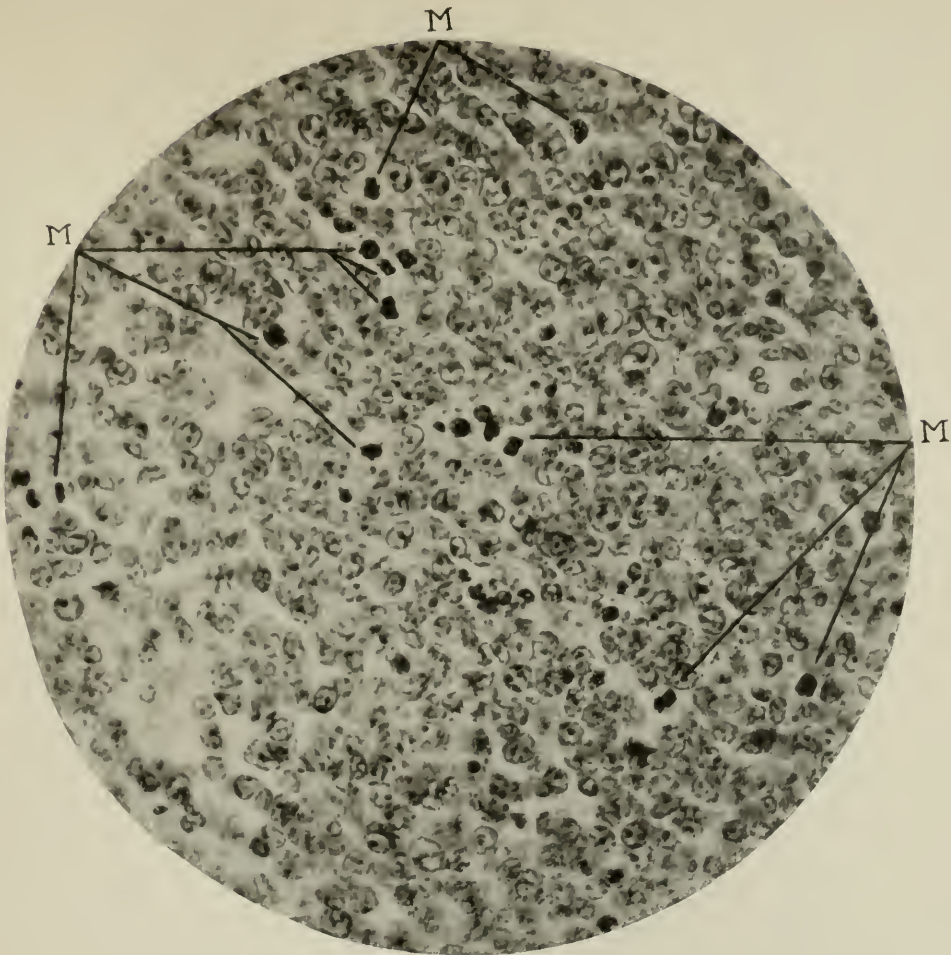


FIG. 5.

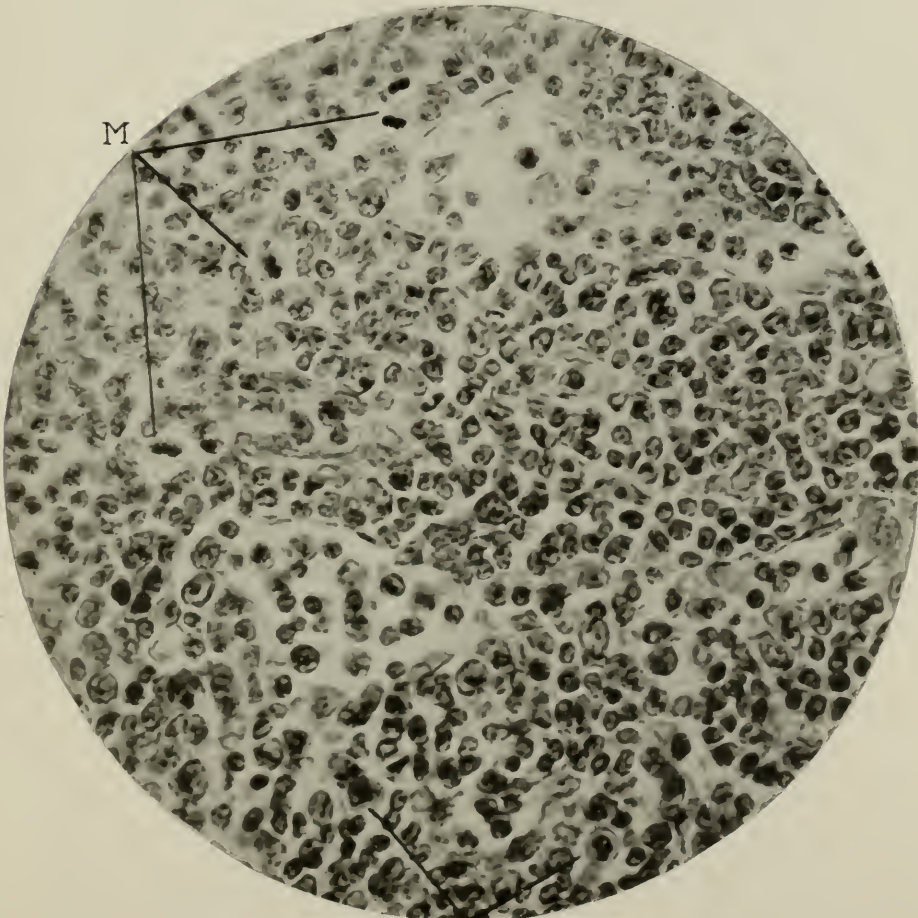


FIG. 6.



THE ERYTHROPOIETIC ACTION OF GERMANIUM DIOXIDE.

II. THE SOURCE OF THE ERYTHROCYTHEMIA PRODUCED BY GERMANIUM DIOXIDE IN THE ALBINO RAT.

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In a previous publication it has been shown that germanium dioxide is non-toxic to the mature female albino rat when injected subcutaneously in amounts up to 180 mg. per kilo of body weight. This is a quantity equivalent to a dose of nearly 11 gm. for a 60 kilo man. The measure of the toxicity was survival after the administration of the compound. In no case did any animal receiving the germanium dioxide succumb or even present obvious symptoms of disturbance.¹

A systematic study was then made of the effect of the compound on the erythrocyte and white cell content of the normal rat blood.² It was found that, regardless of sex, a marked and valid increase in the red cells in the circulation followed the injection of relatively small amounts of germanium dioxide solutions. In view of certain gross findings at autopsy of the rats used in these experiments, and because of the persistence of the effect for many days after the injections, we considered ourselves justified in concluding that germanium dioxide is an erythropoietic agent of remarkable potency.

In this report we propose to present the completed evidence on which that belief is based. The material used was obtained from the rats serving as subjects in the preceding paper.²

¹ Hammett, F. S., Müller, J. H., and Nowrey, J. E., Jr., *J. Pharmacol. and Exp. Therap.*, 1922 (in press).

² Hammett, F. S., Nowrey, J. E., Jr., and Müller, J. H., *J. Exp. Med.*, 1922, xxxv, 173.

A review up to 1915 of the data and literature concerned with the phenomenon of erythrocythemia has been published by Lamson.³

The cause of any given erythrocythemia may be apparent, such as a decrease in the plasma volume, or real, as, for example, a production of new cells, the division of red cells, or a release from deposits. Lamson in studying the erythrocythemia produced by the injection of epinephrine came to the tentative conclusion that the imbibition of fluid by the liver and consequent decrease in plasma volume is a factor of considerable importance.⁴ However, since Lamson and Keith⁵ have reported that the plasma volume and the erythrocyte content of the blood may vary independently of each other, it would appear that their theory has yet to be proved by further experimentation. Because of the incompleteness of the evidence, and in view of our studies of the bone marrow of rats treated with germanium dioxide, we consider that, for the present at least, the question of the relation of the plasma volume to the germanium erythrocythemia is of relatively minor importance.

Similar considerations apply to the question of the possibility of the observed erythrocythemia arising from either a division of red cells already in the circulation or by release from deposits within the organism.

It may be noted here that the germanium dioxide erythrocythemia differs from that produced by Lamson,⁶ with epinephrine in that it is lasting in nature, while that following epinephrine is but transitory. While it is true that we have records of two rats with a persistent red cell increase 5 weeks after a single injection of germanium, and of eleven others showing an erythrocythemia 11 and 14 days after the last injections, yet the objection can be raised, and justly, that this persistence of the effect may well be due to a gradual release of the stimulating compound from a protoplasmically bound condition arising at the time of injection. This would imply a marked stimulating power by relatively minute amounts of germanium. Certain data,

³ Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, 1915, vii, 169.

⁴ Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, 1920-21, xvi, 125.

⁵ Lamson, P. D., and Keith, N. M., *J. Pharmacol. and Exp. Therap.*, 1916, viii, 247.

⁶ Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, 1916-17, ix, 129.

however, indicate that, in man at least, it is necessary to administer a certain minimum amount before a detectable rise in red cells is obtained. This amount is in excess of what might be considered to be released from the tissues during the persistence of effect observed in our studies, per unit of time. This point, however, can only be decided by investigation.

If the erythrocythemia produced by germanium dioxide is actually the result of an erythropoietic action of the compound, evidence should be found in one or all of the blood-forming organs—spleen, liver, and bone marrow—of a stimulation to new red cell formation. Also young erythrocytes should be found in the circulating blood.

Gross inspection of the tissues at autopsy showed that the livers of the germanium-treated rats were of a reddish purple color, while the livers of the controls were a tobacco-brown. The spleen in the rat is normally a very dark red, and no differences in the appearance of this organ in the two groups could be detected. When the marrow of the long bones was exposed it was seen that in the controls this tissue was light coffee color while that of the test animals was maroon.

Small segments of the liver and the spleen were cut from as near the same locality as possible from these organs in all the rats, tests and controls, and were fixed in formaldehyde. The femurs were removed, cracked open so as to expose the marrow, fixed in formaldehyde, and carried through the dehydrating process to absolute alcohol. The marrow was then removed from as near the middle portion of the shaft of the bone as possible and was transferred to chloroform with the other tissues and embedded in paraffin. The animals of any given series were all autopsied within a few minutes of each other, and the fixation, dehydration, and embedding were carried on simultaneously so that errors due to differences in technique would be eliminated.

The sections of the livers and spleens were stained with hematoxylin and eosin. Methylene blue and eosin, and Goodpasture's polychrome stain, without the preliminary permanganate and oxalic acid treatment, were used for the bone marrow.

The sections of the livers of the germanium-treated rats showed no evidence of the resumption by this organ of its original erythropoietic function. There was present, however, in most cases an apparent dilatation of the hepatic capillaries, in which were many erythrocytes

but no nucleated red cells. This relative engorgement of the liver with red corpuscles which did not occur in the controls—and which explains the gross findings at autopsy—rather effectively speaks against the idea that the erythrocythemia is due to liberation from the liver of red cells that might be considered to be held therein, particularly since in the control livers the absence of erythrocytes in the capillaries is noticeable. In fact the increased number of red cells in the livers of the test rats might give rise to the opinion that the actual number of circulating cells observed was not a complete index of the new red cell formation. However that may be, it is certain that, in so far as these observations go, the liver is not the organ causing the erythrocythemia, although it is in some way as yet unknown affected directly or indirectly by the administration of the germanium dioxide.

Study of the sections of the spleens from the test animals also shows no evidence of the taking on of the original function of red cell formation. Nor are there any indications of an increased red cell destruction by the splenic phagocytes. Whether or not an increased red cell destruction accompanies the germanium erythrocythemia can only be determined by investigation. The impression is given that there is a slightly greater erythrocyte congestion in the sections of this tissue from the germanium-treated rats as compared with the controls, but it is not marked enough to be decisive. There also seems to be a more dense concentration of cells in the Malpighian corpuscles of the spleens of the test rats. However, this too is not sufficiently definite to be decisive. It is thus evident that the spleen is not the source of the increased red cell content of the circulating blood, although possibly the content is affected in some way or other, either directly or indirectly, by the administration of germanium dioxide.

In the sections of bone marrow of the germanium-treated rats there was ample evidence that the compound had produced a stimulation in formation of new red cells in that tissue over and above that present in the marrow of the controls. Not only were there more centers where the nucleated erythrocytes were in evidence, but there were more of these types of cells per unit area. Counts were made of the nucleated red cells in twenty fields, under the oil immersion lens, of longitudinal sections cut through the greatest diameter of the pieces

TABLE I.

Counts of the Nucleated Erythrocytes of the Bone Marrow.

Series No.	Sex.	Controls.	Test rats.			
			No. 1.	No. 2.	No. 3.	Mean.
2	Males.	187	242	232		237
	Females.	231	532	620	524	559
3	Males.	254	476	545	611	544
	Females.	308	477	448	492	472

removed from the bones. The field for counting was defined by an eyepiece micrometer. The results are given in Table I. They demonstrate conclusively that the erythrocythemia produced by the injection of germanium dioxide solution takes its origin from a stimulation of erythrocyte formation in the bone marrow.

With such a marked erythrocyte formation one would expect to find evidence in the circulating blood of young red cells. In fact, the presence of such cells in the blood would be a satisfactory confirmation of the bone marrow findings. In none of the rats used in these experiments, tests or controls, could there be found in the blood any cells that could be identified as nucleated erythrocytes. Nevertheless, in the smears from both the controls and the germanium-treated rats there were many polychromatic staining erythrocytes,

TABLE II.

Relative Number of Polychromatic Staining Erythrocytes in the Smears.

	Preliminary period.		During germanium administration.	
	Controls.	Test rats.	Controls.	Test rats.
Mean.....	49.0	40.0	59.0*	79.0
Standard deviation.....	6.4	16.2	12.9	17.6
Probable error of mean.....	1.6	2.4	2.8	2.1

* This slight increase which is by no means comparable with that obtained with the test animals confirms the slight rise in erythrocytes in these animals reported in the preceding paper.²

or young red cells. In order to determine whether or not there were more of these cells in the blood of the test animals as compared with the controls, a systematic count was made of their occurrence in the smears made for the differential leucocyte counts. The results are given in Table II. They represent the means, the standard deviations, and the probable error of the means of all the smears. It should be noted that every time the blood was taken for counting as reported in the preceding paper² smears were also taken for this purpose.

This table shows that there is a statistically valid increase in the number of erythrocytes taking the polychromatic stain in the blood of the germanium-treated rats. This confirms the bone marrow findings.

For purposes of completeness differential counts were made on the smears of all the rats. The normal values are to be published elsewhere. A comparison of the distribution of the white cells of the test rats during the preliminary period with that occurring during the germanium administration and with the controls showed that no changes occurred of sufficient magnitude to be significant.

SUMMARY AND CONCLUSIONS.

A histological comparison of the liver, spleen, bone marrow, circulating young erythrocytes, and differential count in mature male and female albino rats receiving germanium dioxide with their litter controls not receiving this compound was made.

It was found that the livers of the test animals in most cases showed a condition of capillary dilatation and that more erythrocytes were in these capillaries than were in those of the controls. There was no evidence of any red cell formation by the liver.

The spleens of the test rats gave the impression of being slightly more congested and of having a slightly more dense concentration of cells in the Malpighian corpuscles than those of the controls. There was no evidence of an increased red cell destruction nor was there any evidence of splenic erythropoiesis.

In the bone marrow of the rats which had received the germanium dioxide injections there was evidence of a marked stimulation in formation of nucleated erythrocytes, in that many more of these cells were found here than in the marrow sections of the controls.

The circulating blood of the test rats contained more young red cells as demonstrated by the increased number of erythrocytes taking the polychromatic stain than did the blood of the controls.

No noteworthy differences in the values for the various types of white cells in the circulation determined by the differential count could be found between the two groups.

Using, then, as an acceptable criterion of erythropoiesis an increase in the number of erythrocytes in the circulation which is accompanied by an increase in the number of young red cells, and an increased number of nucleated erythrocytes in the bone marrow, we consider ourselves justified in concluding that germanium dioxide is a potent erythropoietic agent and the source of the erythrocythemia produced by this compound is the increased production of red cell precursors by the bone marrow stimulated to increased activity by the compound used.

THE EFFECT OF FLOOD DIURESIS ON HEMOGLOBINURIA.

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The fact is well recognized that a considerable quantity of hemoglobin must be free in the plasma if any is to pass the renal barrier and appear in the urine. The pigment is, like dextrose, a "threshold substance." It readily penetrates into the renal tubules but is absorbed again more or less completely during its course through them.¹ This being true, diuresis should diminish the chances of absorption by hastening the flow of fluid, and tend to lead to the appearance of the pigment in the urine. Evidence will here be presented that such is the case. Hemoglobinuria, like glycosuria, is much favored by flood diuresis.

Method.

A concentrated solution of hemoglobin was abruptly thrown into the circulation of rabbits and dogs, followed in some instances by a slower injection of salt solution. The amount of pigment introduced was slightly less than that required to produce hemoglobinuria in the absence of diuresis. The urine was collected at intervals by catheter.

All of the animals were males. Individuals were selected with normal kidneys, as indicated by the general character of the urine and proven by the autopsy findings.

Great care was necessary to prevent hemorrhage during the catheterization of the rabbits, and despite it a few red cells were frequently encountered afterwards in the urine. For this reason the experiments were repeated on dogs, in which the complication can be avoided. The animals were stretched out, the bladders emptied by catheter as completely as possible, and the urine set aside for

¹ Adami, J. G., *J. Physiol.*, 1885, vi, 382.

TABLE I.
Influence of Flood Diuresis on Hemoglobinuria.

Experiment No.	Rabbit.	Weight.	Hemo- globin of blood.	Per cent of total hemo- globin injected.	Amount injected.	Fol- lowed by salt solution.	Urine of next 4 hr.				Remarks.	
							Amount.	Color.	Guaiaac test.	Spectro- scopic bands.		
		gm.	per cent		cc.	cc.						
1	Control.	3,050	61.5	0.8	1.68	—	3.0	Yellow.	0	Absent.	{ In Experiment 1, the bloods of the animals were as- sumed to have the same percentage of hemoglobin, and in consequence the amounts of pigment in- jected differed directly as the body weight.	
	Diuresis.	2,575	66.5	0.8	1.42	40	26.0	Pink.	+++	Present.		
2	Control.	3,050	71.0	0.8	1.9	—	4.0	Straw.	Tr.	"		
	Diuresis.	2,725	75.0	0.8	1.8	20	8.0	Pink.	+++	"		
3	Control.	2,350	65.0	0.8	1.3	—	1.5	Straw.	0	Absent.		
	Diuresis.	2,450	73.0	0.8	1.6	20	8.0	Pink.	+++	"		
Dogs. 1	Time.											A rather dilute solution of hemoglobin was employed.
	—											
2	Next day.	7,250	72.0	1.6	5.8	—	2.5	Yellow.	0	"		
				1.6	5.8	80	18.0	Orange-pink.	+++	Present.		
	—	5,750	105.0	1.6	15.8	100	93.0	Pinky yellow.	+++	"		
	2 days later.			1.6	15.8	—	15.5	Yellow.	0	Absent.		

3	—	7,250	73.0	1.8	9.6	40	22.0	Port wine.	+++	Present.	{ A different specimen of hemoglobin was used in this first observation from that employed in the three that followed. gr. of morphine sulfate given prior to these observations.
	7 days later.	After 5 days more.	82.0	1.8	5.75	—	12.0	Yellow.	0	Absent.	
	After 3 days more.			1.8	6.5	—	9.0	"	0	"	
				1.8	6.5	100	64.0	Orange-pink.	+++	Present.	

The urines were all negative for hemoglobin prior to the injections; and at no time were red cells found.

test, and the injection made into an ear vein. A hemoglobin solution prepared by the method of Sellards and Minot² from the blood of rabbits was rapidly injected and followed in many instances by warmed 0.9 per cent salt solution. To rule out all possibility of a mistake in the composition of the latter solution such as might, by intravascular laking, cause hemoglobinuria, its effect was tested regularly on an erythrocyte suspension.

A table will be used to present the results (Table I). The amount of hemoglobin injected is there recorded in percentages of the total quantity of the pigment already in circulation, as calculated from the percentage in the blood (Palmer method) and the total blood quantity, assuming this to constitute 5.5 per cent of the body weight in the rabbit and 8 per cent in the dog. Trial showed that in the rabbit one can inject hemoglobin up to at least 0.8 per cent of the body quantity without the appearance of any in the urine, save when diuresis is induced. In the dog 1.6 to 1.8 per cent gives similar results. No attempt was made to determine the normal renal thresholds more closely. In each experiment with rabbits two individuals received the same preparation of hemoglobin in identical proportion, one animal serving as control while in the other diuresis was induced by the injection of 20 to 40 cc. of saline solution given over a period of several minutes. In the work with dogs, each animal served as its own control, receiving repeated injections.

Catheterization was done just prior to each injection and $\frac{1}{2}$ hour after it. The guaiac test and the spectroscope were used to disclose hemoglobin, but in many instances more striking testimony was obtained in the bright pink or red hue of the urine. To determine whether hemorrhage had been a factor, erythrocytes and their shadows were sought for in the urinary sediment.

Nine pairs of rabbits were used, each pair closely matched as regards size and blood condition. In two pairs which received 0.6 per cent and 0.8 per cent of hemoglobin, the urine of all of the animals remained free from the pigment; but only a questionable diuresis had been induced. In a third pair diuresis yielded a red urine, whereas that from the control animal was pink; but both specimens showed fairly numerous erythrocytes. In the remaining six experiments diuresis regularly resulted in a pink or red urine containing much hemoglobin and free from red cells or shadows, in contrast to the specimens from the controls, which, though sometimes showing a few red cells, were always yellow and only once yielded a positive guaiac reaction after the cells had been removed. Three such sharp-cut instances are presented in the table.

Four dogs were used. In one the results were relative only, hemoglobin appearing in quantity when diuresis was induced, and to a less extent next day when diuresis was lacking after the pigment injection. The findings in the remaining three animals have been tabulated (Table I). It will be seen that diuresis regularly resulted in hemoglobinuria, whereas without it none occurred.

² Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917-18, xxxvii, 161.

By varying the order of the experiment, an overloading of the organism with pigment was ruled out as responsible for the results.

It is evident that flood diuresis markedly influences the elimination of hemoglobin into the urine, lowering the renal threshold for the substance as it does that for dextrose. According to Cushny³ flood diuresis probably never occurs in man as a result of fluid taken by mouth. The tubules of the rabbit kidney are much less active in resorption than those of human beings; yet when very large quantities of water are administered to this animal "even its feeble power of absorption is sufficient to save the optimal fluid."³ The present findings, then, have no bearing on the occurrence of clinical hemoglobinuria. They are not without significance, however, for a proper understanding of the renal siderosis that occurs in diseases which involve the repeated liberation of small quantities of blood pigment into the circulation.

CONCLUSIONS.

Flood diuresis so far lowers the renal threshold for hemoglobin that the pigment appears in quantity in the urine as result of a hemoglobinemia insufficient under ordinary circumstances to lead to the elimination of even a trace of it. In pathological conditions that involve blood destruction hemoglobin probably passes into the tubules much more often than it reaches the urine, being prevented therefrom by the resorptive activity of the tubular epithelium.

³ Cushny, A. R., *The secretion of the urine*, London, New York, Bombay, Calcutta, and Madras, 1917, 145.

SIGNIFICANCE OF THE HEMOSIDEROSIS OF PERNICIOUS ANEMIA.

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The view that pernicious anemia is due to an injurious agent derived from the gastrointestinal tract has of late years attained the proportions of a doctrine, and it has determined the direction of all recent efforts to find the cause of the disease. Whether such a view is inevitable is a question worth asking. In the present paper we shall show that one of the findings which has been considered well-nigh conclusive in its support is in reality worth little as evidence. We refer to the marked siderosis of the liver parenchyma that occurs during pernicious anemia, a localization of pigment which has been taken to indicate that pathological blood destruction is localized within the portal tributaries.

Hunter ¹⁻⁶ in special has laid emphasis on the difference in distribution of the hemosiderin found in the organs of cases of pernicious anemia and that laid down as the result of frank blood destruction throughout the circulation as a whole. Siderosis in instances of the latter sort is usually far more marked in the kidneys and spleen than in the liver where it is especially abundant in pernicious anemia. Hunter's contention that a portal blood destruction is thus indicated in pernicious anemia receives support from the quite different distribution of the siderosis in animals in which hemolysis has been experimentally induced by injections into

¹ Hunter, W., *Lancet*, 1888, ii, 556.

² Hunter, W., *Lancet*, 1888, ii, 608.

³ Hunter, W., *Lancet*, 1888, ii, 654.

⁴ Hunter, W., *Lancet*, 1903, i, 283.

⁵ Hunter, W., *Lancet*, 1903, i, 367.

⁶ Hunter, W., *Pernicious anæmia: its pathology, septic origin, symptoms, diagnosis, and treatment*, London, 1901.

the systemic circulation^{3, 6, 7, 8}, and in those given intravenous or subcutaneous injections of hemoglobin. Schurig,⁹ for example, found that daily injections of the hemoglobin of the horse into the subcutaneous tissue of rabbits led to an abundant siderosis of the kidneys with but a slight one of the liver.

Recent work on the removal of hemoglobin from the plasma by the liver and kidneys has suggested to us the possibility that differences in siderosis such as those described might be produced merely by varying the amount of pigment set free into the general circulation. We shall record experiments which prove that this is the case and then discuss the theoretical considerations leading to and deriving from them.

Method.

Young rabbits, mostly of less than 1,500 gm. weight have been used for the work. The liver of old normal animals of many species contains not infrequently a few granules of hemosiderin, but in immature rabbits of the size indicated they are not found. The hemoglobin was prepared from rabbit corpuscles by the method of Sellards and Minot.¹⁰ It was given in concentrated watery solution of 120 to 160 per cent hemoglobin strength (Palmer) into the subcutaneous tissue of the flanks and abdomen, care being taken to select spots where there were no large vessels; and gentle massage was done to diffuse the pigment somewhat, but not greatly, since our aim was to bring about a gradual absorption throughout the 24 hours. We were able in many instances to assure ourselves that this actually happened, by direct inspection from time to time of the injection area through the translucent skin. Injections were given on 6 days out of every 7. For purposes of tabulation (Table I) the daily hemoglobin dose is expressed roughly, in terms of the total hemoglobin of the blood of each individual, as calculated from the hemoglobin percentage (Palmer), and the total blood quantity, assuming the latter to constitute 5.5 per cent of the gross body weight. There were no great differences in the hemoglobin percentages of the animals selected, so the only important variable was the weight. Many of the rabbits grew so rapidly as to necessitate frequent increases in the amount of pigment given. The injection period ranged from 13 to 102 days and the hemoglobin dose from $\frac{1}{4}$ that normally possessed by the animal to $\frac{1}{150}$ of it. The larger injections caused some loss of body weight, but in general the health of the animals was remarkably good.

⁷ Hunter, W., *Severest anæmias; their infective nature, diagnosis and treatment*, London, 1909.

⁸ Muir, R., and Dunn, J. S., *J. Path. and Bact.*, 1914-15, xix, 417.

⁹ Schurig, *Arch. exp. Path. u. Pharmacol.*, 1898, xli, 29.

¹⁰ Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917-18, xxxvii, 161.

The principal complication developing was a hemoglobinuria from the summated effect of the injections. Haessler¹¹ has noted that in the rabbit hemoglobinuria follows the rapid introduction into a vein of pigment amounts greater than $\frac{1}{125}$ of that possessed by the animal. Frequently in our experience, when a considerable amount of pigment had been introduced beneath the skin all was not absorbed before the next injection, and after a number of injections absorption would be going on from several different regions at once with result that pigment appeared in the urine. When small amounts were used, only a slight brownish stain could be detected in the subcutaneous tissue after 24 hours, yet even in such instances the urine tests were often eventually positive for hemoglobin. Whether this was due to an overtasking of the abilities of the other organs removing pigment from the blood, or resulted from changed local conditions we have not inquired. Induration at the site of injection rarely occurred.

The urine of most of our rabbits was daily submitted to the guaiac test, though, as is well known, a positive reaction does not always mean hemoglobinuria in these animals, and we were but seldom able to demonstrate blood pigment with the spectroscope. That it was actually present in the urine in many of the positive instances was indicated by their time of occurrence, which was after several large injections or a considerable number of small ones.

When large amounts of hemoglobin had been given, the renal cortex proved to be snuff-colored from siderosis, but the color of the liver was never distinctly altered from the normal, and the spleen was quite unenlarged and unchanged in hue. Chloroform was the routine lethal agent. Blocks from the liver, kidney, spleen, and red bone marrow were fixed in alcohol and stained with ammonium sulfide, and in duplicate, by the combined ammonium sulfide-potassium ferrocyanide method of Nishimura. Muir and Dunn⁸ advocate the use of hot hydrochloric acid in carrying out Nishimura's technique, and, confirming their results, we find that iron is much more sharply demonstrated thereby. But since practically all of the data on the distribution of hemosiderin in human beings have been obtained by the less perfect methods above mentioned, we have given most attention to their use. The criterion used in gauging the degree of siderosis was likewise that employed with most of these data, namely the appearance of the tissue under the microscope. Since only special cells and regions of each organ undergo pigmentation, a decision as to which organ contains most hemosiderin cannot be reached, unless the differences are pronounced.

RESULTS.

The distribution of hemosiderin, as ascertained by the means described, differed markedly with the amount of hemoglobin given (Table I). When a daily portion of less than $\frac{1}{30}$ of the approximate quantity of pigment in circulation was employed, practically no

¹¹ Haessler, H., *J. Exp. Med.*, 1922, xxxv, 515.

TABLE I.

Relative Siderosis of the Rabbit Liver and Kidneys.

Rabbit No.	Final weight.	Proportion of animal's own hemoglobin given each time.	Period covered by injections.	Observed siderosis.	Remarks.
	<i>gm.</i>		<i>days</i>		
1	1,750	$\frac{1}{150}$	80	None.	No tests made for hemoglobinuria.
2	1,850	$\frac{1}{120}$	80	"	No tests made for hemoglobinuria.
3	1,600	$\frac{1}{120}$	37	Very slight; more in liver than kidneys.	Guaiac test occasionally positive; spectroscopic findings negative.
4	1,800	$\frac{1}{100}$	102	None.	No tests made for hemoglobinuria.
5	2,100	$\frac{1}{100}$	68	"	No tests made for hemoglobinuria.
6	1,550	$\frac{1}{90}$	37	Much of liver; none of kidneys.	Guaiac test occasionally positive; spectroscopic findings negative.
7	1,650	$\frac{1}{80}$	72	Much of liver; a few granules in kidneys.	No tests made for hemoglobinuria.
8	1,500	$\frac{1}{75}$	37	Much of liver; some of kidneys.	Old scarring of kidneys in both instances. Guaiac test occasionally positive; spectroscopic findings negative.
9	1,650	$\frac{1}{60}$	37	Much of liver; some of kidneys.	
10	1,650	$\frac{1}{30}$	20	Marked kidney siderosis; little of liver.	No hemoglobin ever in urine.
11	1,375	$\frac{1}{30}$	19	Similar amount in both organs.	Hemoglobin demonstrated in urine with the spectroscope.
12	1,350	$\frac{1}{20}$	18	Much kidney siderosis; little of liver.	Hemoglobin found once with the spectroscope.
13	2,000	$\frac{1}{8}$	13	Very marked kidney siderosis; little of liver.	Hemoglobin often in the urine as shown with the spectroscope.
14	1,750	$\frac{1}{4}$	13	Very marked kidney siderosis; little of liver.	Hemoglobin almost regularly in the urine.

siderosis was anywhere to be found, even after the injections had been kept up for many weeks. By the use of hot hydrochloric acid a blue dot could sometimes be demonstrated within the nucleus of many cells of the convoluted tubules of the kidneys, but with the ordinary technique it was not evident and so may be dismissed from attention. After slightly larger injections, long continued, the liver parenchyma exhibited a well defined stippling with hemosiderin, which, like that of pernicious anemia, was most pronounced near the periphery of the lobules; whereas the kidneys were non-pigmented, or negligibly so. With still more hemoglobin, the differences in the organs became less noteworthy and sometimes the kidney tubules showed an equal or greater siderosis. Always when large injections had been given, resulting in hemoglobinuria after but a few days, the epithelium of the renal tubules was heavily siderosed with coarse granules or lumps, and the hepatic parenchyma was by contrast negligibly pigmented. Often in such instances an amorphous material giving the iron reaction was to be seen lying free in the glomerular capsule and the lumen of the upper tubules, a material such as Muir and Dunn^{8,12} noted after intravascular hemolysis. In the spleen no special siderosis was ever found, thus bearing out Schurig's contention that this organ, while a great locus of deposit for injured cells, has no special power to retain the pigment derived from them. The same would seem to be true of the bone marrow, to judge from our specimens.

An attempt was made to repeat the work with dogs, but several obstacles intervened that proved decisive. Dog hemoglobin has a marked tendency to crystallize out of solution. We were unable to keep it for more than a few hours in concentrations greater than 55 per cent (Palmer); and to administer the necessary amounts in dilute form entailed large injections that were often ill-borne. Furthermore, the relative vascularity of the subcutaneous tissue of the dog, as compared with that of the rabbit, and the wide extension of the injected material through it, combined to result in such rapid absorption that hemoglobinuria was rendered frequent. On the other hand, when the pigment had not been spread by massage, abscesses often developed. In the dog, too, the Kupffer cells have what would seem

¹² Muir, R., and Dunn, J. S., *J. Path. and Bact.*, 1915-16, xx, 41.

to be an unique predilection for hemoglobin. In several of our animals the liver sections submitted to the ferrocyanide reaction, appeared deep blue to the unaided eye, obviously containing enormous quantities of iron, but with the microscope this iron was seen to be confined almost wholly to the Kupffer cells which showed both a granular and a diffuse coloration. The six young dogs of our experiments were given hemoglobin in amounts of from $\frac{1}{80}$ to $\frac{1}{10}$ of that possessed in the blood. All ultimately developed hemoglobinuria and were sacrificed for that reason. The findings as regards relative siderosis of the liver and kidney parenchyma were inconclusive, as might have been predicted from these facts.

The renal siderosis in both the dog and rabbit affected especially the cells of the proximal convoluted tubules and of the ascending limb of Henle's loop, as is the case in pernicious anemia, but not in hemochromatosis,¹³ a disease in which there is no evidence of pathological blood destruction.

DISCUSSION.

We have shown that the constant presence in the general circulation, as distinct from the specifically portal, of a small amount of free hemoglobin leads eventually to a siderosis of the liver similar to that which has been considered so significant in pernicious anemia. When this amount is kept within certain limits, renal siderosis fails to appear or is negligible in degree. When more is given, the epithelium of the renal tubules rapidly becomes pigmented, the iron deposition far outstripping that in the liver. These are the facts. The conception which led to the experiments demonstrating them,—and which itself receives strong support from them,—will now be outlined. It is based on the differing activities of the liver and kidneys in the elimination of hemoglobin.

The liver possesses a special ability to remove free hemoglobin from the blood stream,¹⁴ and under normal circumstances is probably the principal organ, if not the only one, that keeps the plasma free from the pigment. It may still succeed in so doing,—and in the

¹³ Gaskell, J. F., Sladden, A. F., Wallis, R. L. M., Vaile, P. T., and Garrod, A. E., *Quart. J. Med.*, 1913-14, vii, 129.

¹⁴ Dubin, H., and Pearce, R. M., *J. Exp. Med.*, 1917, xxv, 675.

doing become siderosed—when there is a persistent slight increase in the amount of hemoglobin coming to it. Its ability, though, is overtasked by any considerable frank hemolysis,—as is often evidenced by the hemoglobinuria then ensuing; and when this is the case, the pigment accumulates in greater or less quantity in the blood, to be dealt with by the kidneys according to the laws governing the fate of threshold substances. Hemoglobin is, like dextrose and many foreign dyestuffs, a threshold substance in its renal relations,¹⁵ one which readily passes the glomerular barrier but undergoes a greater or less resorption during its passage through the tubules. One would suppose, *a priori*, that hastening this passage would favor hemoglobinuria by lessening the opportunities for resorption, just as glycosuria is favored. And, indeed, Haessler,¹¹ in a paper from this laboratory, has demonstrated the fact that flood diuresis causes marked hemoglobinuria, when it is induced immediately after the intravenous injection of an amount of blood pigment so small that none passes into the urine under ordinary circumstances. All this being true, it is obvious that there must be many clinical occasions when hemoglobin passes into the tubules but fails to reach the urine, owing to the completeness of its resorption. A similar resorption of foreign dyestuffs leads to their deposition in quantity in the tubule cells. Does not renal siderosis come about in a like manner? And, when there is much blood pigment to be resorbed, will not the unusual opportunities of the tubule cells to obtain it lead to a noteworthy hemosiderin deposition within them? The results of our experiments indicate that these questions are to be answered in the affirmative. The findings do not permit of a conclusion as to whether the freedom of the kidney from hemosiderin when small amounts of hemoglobin have been administered is due to a glomerular threshold for the latter substance, as distinct from the greater renal one. Not infrequently, after large injections of hemoglobin, the amorphous contents of the glomerular capsule and tubules yields a positive reaction for iron, a fact which strongly suggests that hemosiderin may circulate as such in the blood and be excreted in the urine where indeed one of us has already found it lying free in granular form.¹⁶

¹⁵ Cushny, A. R., *The secretion of the urine*, London, New York, Bombay, Calcutta, and Madras, 1917.

¹⁶ Rous, P., *J. Exp. Med.*, 1918, xxviii, 645.

TABLE II.

*Relative Siderosis of the Liver and Kidney in Pernicious Anemia, Determined Microscopically.**

Author and reference.	No. of cases.	Degree of siderosis.		Technique used.
		Liver.	Kidney.	
Quincke, H., <i>Samml. klin. Vortr.</i> , 1876, No. 100,797.	2	Equal in both.		
	1	Marked.	Very slight.	
Quincke, H., <i>Deutsch. Arch. klin. Med.</i> , 1877, xx, 1.	1	"	None.	Potassium ferrocyanide and hydrochloric acid.
Quincke, H., <i>Deutsch. Arch. klin. Med.</i> , 1879-80, xxv, 567.	2	"	Slight.	Ammonium sulfide.
	2	"	None.	
Mott, F. W., <i>Lancet</i> , 1889, i, 520.	1	"	"	Potassium ferrocyanide and hydrochloric acid; also ammonium sulfide.
Mott, F. W., <i>Lancet</i> , 1890, i, 287; <i>Practitioner</i> , 1890, xlv, 81.	1	"	Slight.	Potassium ferrocyanide and hydrochloric acid; also ammonium sulfide.
Griffith, J. P. C., and Burr, C., <i>Tr. Assn. Am. Phys.</i> , 1891, vi, 239.	1	"	"	Potassium ferrocyanide and hydrochloric acid; also ammonium sulfide.
		"	None.	
Hopkins, F. G., <i>Guy's Hosp. Rep.</i> , 1893-94, i, 349.	5	Equal in both.		Potassium ferrocyanide and hydrochloric acid.
Stühlen, A., <i>Deutsch. Arch. klin. Med.</i> , 1894-95, liv, 248.	1	"	"	
	3	Marked.	Slight.	
	2	Moderate.	None.	
Warthin, A. S., <i>Am. J. Med. Sc.</i> , 1902, cxxiv, 674.	4	Equal in both.		Technique not mentioned.
	4	Marked.	Less.	
Hunter, W., <i>Lancet</i> , 1903, i, 367.	1	Slight.	Moderate.	Potassium ferrocyanide and hydrochloric acid.
	1	Moderate.	Slight.	

* The reports of Hunter's many cases ⁴⁻⁷ do not lend themselves well to tabulation.

TABLE II—*Concluded.*

Author and reference.	No. of cases.	Degree of siderosis.		Technique used.
		Liver.	Kidney.	
Gulland, C. L., and Goodall, A., <i>J. Path. and Bact.</i> , 1905, x, 125.	3	Equal in both.		Potassium ferrocyanide and hydrochloric acid.
	5	Marked.	Moderate.	
	3	"	Slight.	
	4	Present.	None.	
	2	"		
Schneider, J. P., <i>J. Am. Med. Assn.</i> , 1920, lxxiv, 1759.	5	Equal in both.		Technique not mentioned.
	5	Marked.	Moderate.	
	2	"	None.	

The observed variations in the distribution of hemosiderin in pernicious anemia are rendered understandable by the facts just presented. In Table II a summary is given of the records of several observers on siderosis in the disease. It will be seen that while an hepatic pigmentation not infrequently exists without any siderosis of the kidney, and is usually relatively marked when the latter occurs, yet cases are not wanting in which the renal siderosis equalled that in the liver (Table II). Such individual differences in the siderosis of the disease may be due to the same cause that has yielded like findings in the animals of our experiments, *viz.* differences in the amount of free hemoglobin in the general circulation. Livers that are damaged—and the organ is assuredly damaged in pernicious anemia as a fatty change attests—not infrequently become somewhat siderosed in the absence of a pathological blood destruction;¹⁷ and when this is present pigmentation may become extreme.¹⁸

Let it be granted, despite the foregoing, that blood destruction during pernicious anemia is mainly portal. Even this constitutes no reason for the belief that the disease has its seat within the viscera draining to the liver by the portal stream. Too little notice has been taken of the circumstance that red cells, damaged in many body regions and in many different ways, are alike "scrapped"

¹⁷ Kretz, R., *Beitr. klin. Med. u. Chir.*, 1896, No. 15.

¹⁸ Rous, P., and Oliver, J., *J. Exp. Med.*, 1918, xxviii, 629.

within the spleen, whence the liberated hemoglobin passes to the liver. Thus it is with cells damaged by a burn of the skin,¹⁹ by a hemotoxin,²⁰ or specific hemolysin injected into a peripheral vein, by chemical agents such as toluylenediamine,²¹ or by the principles responsible for incompatibility on transfusion. Even under normal conditions, the same local deposition of corpuscular debris goes on,²² as it does also to a much more considerable extent during repair from secondary anemia, when the unusually frail cells put forth by the marrow are threshed to pieces whilst in circulation.²³ All this is to say that many sorts of blood destruction which are systemic in origin become portal in completion. So it may well be with the destruction that takes place during pernicious anemia.

A brief comment upon the other evidence for a portal origin of pernicious anemia will not be out of place. Stress has been laid upon the resemblance between the disease and *Bothriocephalus* anemia, and on the results of experiments whereby a blood picture not dissimilar from that of pernicious anemia has been produced through the action of hemolytic substances absorbed from the gastrointestinal tract. There can be, of course, no doubt that substances affecting a liberation of hemoglobin within the portal vessels will cause a more distinctive liver siderosis than those active within the circulation generally. Yet the latter can suffice as we have shown. The symptoms from the alimentary tract during pernicious anemia, and the anatomical changes ultimately found therein, constitute no stronger evidence on the seat of the disease than do the nervous symptoms and changes. The most that can be said of them is that they are suggestive. But no matter how suggestive they seem in association with the other facts just mentioned, it is surely good policy to box the compass of possibilities in the consideration of so obscure a disease as pernicious anemia, rather than to look for enlightenment in a fixed direction.

¹⁹ Askanazy, M., in Aschoff, L., *Pathologische Anatomie*, Jena, 4th edition, 1919, i, 69.

²⁰ For example, the megatheriolysin of Todd.

²¹ Joannovics, G., *Z. Heilk.*, 1904, xxv, 25.

²² Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651.

²³ Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665.

SUMMARY.

The selective deposition of hemosiderin in the liver parenchyma during pernicious anemia does not constitute evidence that there is a hemolytic cause for the disease located in the portal region. The repeated introduction of small amounts of free hemoglobin into the general circulation, by the subcutaneous route, leads, as we have shown, to an identical siderosis. Larger amounts of hemoglobin cause a renal pigmentation equalling or exceeding the hepatic, a fact that is in keeping with what is known of the physiology of hemoglobin excretion and of the findings in human beings after outspoken hemolysis.

THE RENAL ELIMINATION OF BILIRUBIN.

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The mode of escape through the kidneys of circulating blood and bile pigment has received but scanty attention in the past as compared with that of foreign dyestuffs. These last have been the subject of a multitude of experiments and of controversies that are not yet at an end. The reason for this is plain. The dyestuffs have been utilized for the better discovery of principles which would apply supposedly to the body pigments as well. But since these principles are still ill defined and their general application is unattested, direct studies on the renal elimination of hemoglobin and bilirubin would seem desirable—more especially since patients with these pigments in their urine continue to demand attention.

Authorities agree that the kidney is often severely injured by jaundice. Albuminuria and cylindruria (Nothnagel) appear early. Quincke¹ has comprehensively described the anatomical changes. At an early stage the cortex is diffusely stained with bilirubin. As time passes the pigment accumulates in granular form in the cells of the convoluted tubules and more markedly in those of the loop of Henle, and in the lumen of the latter many free granules may be seen, together with yellow, green, or brown casts. It is noteworthy that the glomeruli remain practically unstained. There is cloudy swelling of the tubular epithelium, with loss of the brush border, and even necrosis here and there. In Quincke's opinion, these severe changes cannot but result in a lessened renal activity, and thus may have serious consequences for the organism as a whole.

Significance of Jaundiced Cells in the Urine.

According to text-books on clinical microscopy a "bile-stained urinary sediment" is a regular accompaniment of marked jaundice. It has been tacitly assumed that the bile staining is a staining of dead

¹ Quincke, H., in Nothnagel, H., *Specielle Pathologie und Therapie*, Vienna, 1899, xviii, 63.

cells by the fluid in which they lie immersed, and is devoid, therefore, of clinical significance. Such is often the case. But there is another possibility, that some of the cells may be kidney cells impregnated with pigment prior to desquamation into the urine. Such cells would afford a direct index to the renal condition, like the cells containing granular hemosiderin that are to be found in the urine of patients with hemochromatosis and pernicious anemia.²

In the urine of icterus neonatorum, cells containing granular and crystalline bilirubin are almost regularly present, often indeed when no dissolved pigment can be demonstrated. An excellent paper on the theme is that of Cruse³ whose technique for the microscopic Gmelin reaction we have found highly useful. No systematic observations have been reported on the urinary cells of jaundiced adults, or at least none carried out from the standpoint of kidney physiology.

We have studied the urinary sediment of many icteric human beings and of dogs with jaundice produced in several ways—by fasting, by blood destruction with toluylenediamine or a specific hemolytic serum, and by ligation of the common duct. Two types of cell staining with bilirubin have been discriminated, one consequent on sojourn of the elements in the urine, the other a direct expression of the renal condition. In the one the cells from all parts of the urinary tract are stained, whereas in the other, no matter how deep the jaundice, the leucocytes, squamous epithelium, and bladder epithelium are uncolored, whereas the cells of manifest renal origin may be deeply stained. Needless to say, both types of staining, or only that first mentioned, will be seen in urines that have stood for a long time. The specific pigmentation of the renal cells is most evident in specimens freshly voided; and some hours are required for the bilirubin to dissolve out of the more heavily impregnated cells. After long continued jaundice in man, the urinary sediment yields striking indications of the serious condition of the kidneys. Numerous cells, often disintegrating, will be observed, crowded with coarse, opaque, brown granules or irregular particles, and many such particles lie free. All give under the microscope an intense Gmelin reaction. The pigment cannot be confused with the yellowish lipoidal substances

² Rous, P., *J. Exp. Med.*, 1918, xxviii, 645.

³ Cruse, P., *Arch. Kinderheilk.*, 1880, i, 353.

found in some pathological states.⁴ Intracellular clumps of narrow brown needles are frequently present. The cytoplasm of the renal cells is usually stained deep yellow, though not always. The desquamated elements from the lower urinary tract are by contrast practically colorless, though lying in a dark, icteric fluid.

Special interest attaches to the findings in subicteric states and during slight and transient jaundice. Bile pigment is a threshold substance in human beings, one which readily passes into the tubules but is absorbed again in its course through them, and so rapidly that often none can be found in the urine when a considerable quantity exists in the blood. Will the cast off renal cells yield evidence of this absorption process when the plasma contains bile pigment but tests fail to disclose it in the urine? Such has not proved to be the case. In urines that fail to give the Gmelin reaction, cells stained with bilirubin are absent. Evidently there must be in human beings a special glomerular threshold for bilirubin as well as a more general, higher one for the kidney as a whole. For were this not so, the renal epithelium found in the urine should be tinted with bilirubin whenever the plasma is colored with it—and the plasma is so colored normally.

Human urine during slight or transient jaundice regularly contains renal cells tinted a diffuse yellow and yielding the Gmelin reaction, in contrast to the colorless and negatively reacting elements of the lower tract. Granular bilirubin is not seen. We are inclined to believe that Rosenbach's method of test,⁵ whereby much urine is passed through a filter and this latter submitted to the Gmelin reaction owes its delicacy, in part at least, to the accumulation upon the paper of specifically stained cells.

In dogs the slightest and most transient jaundice may lead to an output of renal cells brilliantly stippled with bilirubin. The pigment occurs as small or coarse, rounded or oblong, granules of a bright mahogany-brown, scattered irregularly in the ground glass cytoplasm of large cells with a rather small, rounded nucleus. The cytoplasm itself is usually unstained save sometimes for a very distinct brownish red zone, or halo, around each granule. In kept specimens such

⁴ Weicksel, J., *Deutsch. Arch. klin. Med.*, 1919, cxxx, 260.

⁵ Rosenbach, O., *Centr. med. Wissensch.*, 1876, xiv, 5.

halos of dissolved bilirubin regularly develop. These and the ruddy tint of the pigment go far to differentiate the latter from hemosiderin in the absence of chemical tests. When the urine stands for 24 hours at room temperature, the brown granulation usually disappears but in the ice box it persists much longer. Severe jaundice is accompanied in both dogs and rabbits by heavily granulated cells and free particles of bilirubin in the urine, just as in the case of human beings.

It is a curious fact that many dogs with slight jaundice yield only diffusely tinted renal cells, whereas in others with no greater icterus, elements stippled with bilirubin are regularly encountered. This difference is consistently maintained over considerable periods of time. We have noted it day after day in catheterized specimens from animals possessing normal kidneys, and with "physiological icterus" induced by fasting. It is independent of the reaction of the urine or of diuresis. Quite possibly the difference is one in derivation of the cells, those from the stippled portions of the tubules failing to desquamate in some animals.

Effects of Flood Diuresis in Dogs.

The sources of damage to the kidneys during jaundice have never been precisely determined. The excretion of bile salts may be, and probably is, far more injurious than that of bile pigment, yet there is no doubt that the accumulation of the latter in the renal cortex, as in the system generally, should be avoided if possible. Diuresis has long been advocated for the purpose. We have tested out its efficacy upon dogs.

Animals were selected that during a period of several days showed no albuminuria or casts. They were kept in metabolism cages. Under ether, all of the large bile ducts were separately ligated, or the common duct was tied and cut and the neck of the gall bladder similarly obstructed to rule out any influence of this reservoir upon the course of the jaundice. Asepsis was maintained throughout, and the incision was closed in three layers. The accumulation of bilirubin in the blood, together with its output in the 24 hour urine, was carefully followed by means of the quantitative method of van der Bergh and Snapper⁶ for the blood, and Hooper and Whipple's⁷ modification of the Salkowski method for the urine.

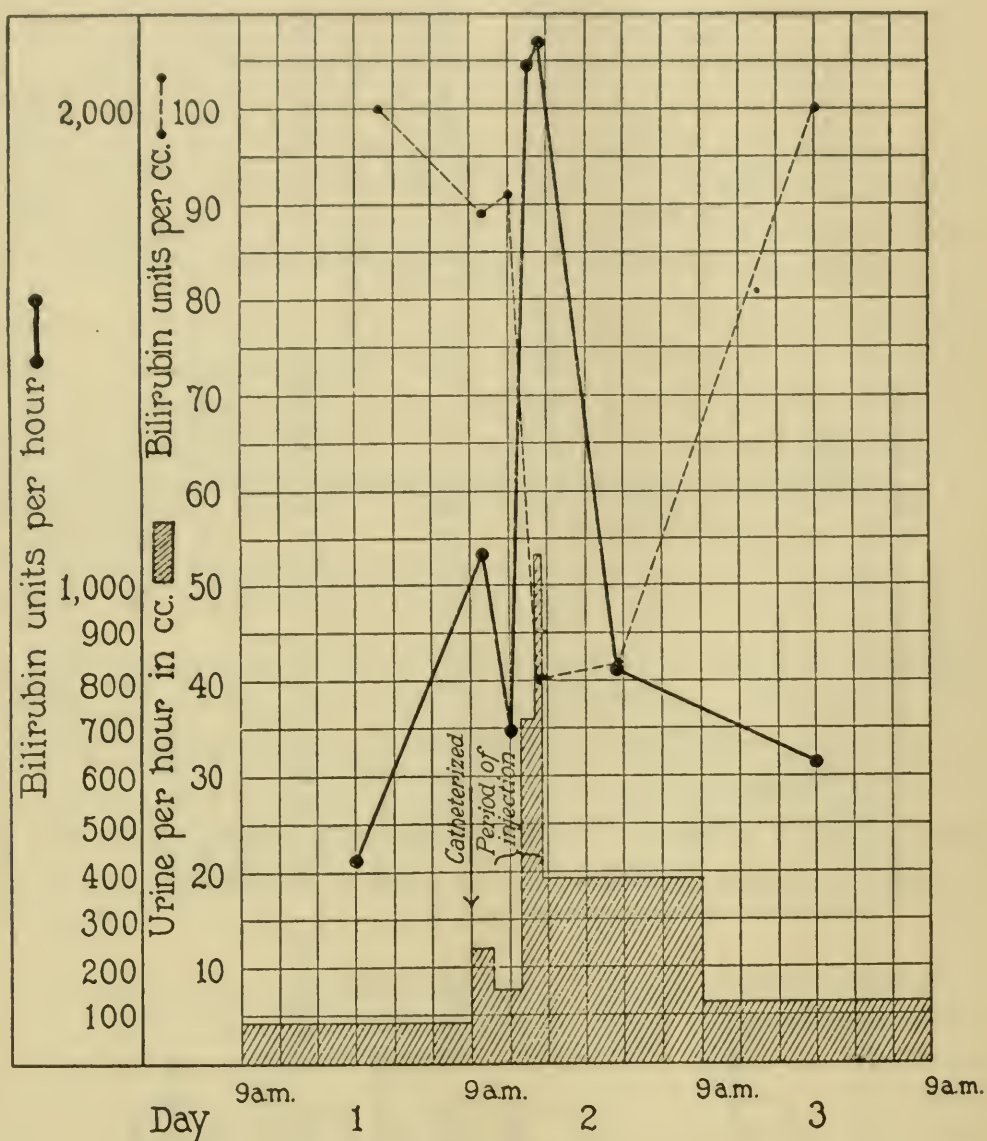
⁶ van der Bergh, A. A. H., and Snapper, J., *Deutsch. Arch. klin. Med.*, 1913, cx, 540.

⁷ Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

TABLE I.
Effect of Flood Diuresis on Bilirubinuria.

Time of occurrence after operation.	Time.	Procedure.	Fluid injected.			Urine.			Bilirubin units.			Blood.	
			Total.	Rate per kilo per min.	Period.	Total.	Output per hr.	Color.	Output per cc.	Total.	Output per hr.	Hemoglobin.	Bilirubin content per 100 cc. of plasma.
days	a.m.		cc.	cc.	hrs.	cc.	cc.					per cent	mg.
18													
19	9.00	Catheterized.			24	100	4.17	Dark brown.	100	10,000	417		
	10.00												
	11.15	Catheterized. Injection begun.		0 37	2½	27	12.0	"	89	2,400	1,067	89	1.47
	11.45	Speed of injection altered.	100	0.17									
	p.m.												
	2.15	Catheterized. Speed of injection altered.	325	0.185	3	23	7.7	Orange.	91	2,090	698		
	3.00		400										
	3.30	Catheterized.	500	0.37	1½	45	36.0	Yellow.	58	2,610	2,090		
	4.00	Injection stopped.	550	0.185									
	4.15	Catheterized.			¾	40	53.3	"	40	1,600	2,133	87	1.33
	a.m.												
20	9.00				∞ 16½	325	19.4	"	42	13,630	814	87	1.72
21	9.00				24	150	6.25	Light brown.	100	15,000	625		

After 10 or more days of obstruction had elapsed and the icterus had reached a relatively constant level, the study of the effects of intravenous injections of fluid was begun. The animal was stretched out; the bladder was emptied with a catheter; and warmed 0.9 per cent sodium chloride solution was introduced into



the experiment. The injection was continued for 3 hours or more, during which time the urine was collected by catheter and its pigment content determined. The experiment was successfully carried out four times in all, on three dogs.

The findings were consistent. The diuresis consequent on the injection of salt solution regularly increased the output of bile pigment greatly. The amount per cubic centimeter of urine, on the other hand, was much lessened, unlike that of hemoglobin, which under similar circumstances⁹ is notably augmented. The intensity of the bilirubinemia remained practically unaffected. A single protocol will suffice to show the findings.

Experiment 1.—A brown female collie weighing 9 kilos was operated upon 19 days before the diuresis experiment and all duct branches were tied, and, where possible, cut. The amount of bilirubin contained in the urine was not measured in milligrams but in terms of an arbitrary standard of units, so called. The findings for this reason are without quantitative significance in relation to the bilirubin content of the blood. Table I gives the course and results of the experiment and Text-fig. 1 illustrates it graphically.

Effects in Dogs of Diuresis from Water by Mouth.

The effects of large amounts of water administered by mouth to dogs with the jaundice of total obstruction were followed in several series of animals.

Dogs tolerate total biliary obstruction for many weeks but lose appetite, drink little, and gradually emaciate and become anemic. The kidneys undergo a progressive injury. For these reasons it was deemed best to begin the observations within a few days after the ducts had been obstructed at operation, and by alternating periods of forced fluid by mouth with those in which there was only the normal intake to render each animal its own control. Dogs with normal blood and urine were selected for the work. Since the character and amount of the food are supposed to influence the quantity of bile pigment formed¹⁰ a constant ration of bread and meat was supplied each day and the amount taken was determined. Fortunately the animals ate about as much when water was forced as during the control periods.

Much difficulty was experienced in determining the precise amount of pigment eliminated in the urine from day to day, more especially during the periods of diuresis. The following modification of Hooper and Whipple's method was

⁹ Haessler, H., *J. Exp. Med.*, 1921, xxxv, 515.

¹⁰ Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349.

finally employed. 2 per cent of the 24 hour specimen was made up to 20 cc. with water, alkalized with sodium carbonate, the bilirubin precipitated out with calcium chloride as usual, and the bluish green coloration obtained with acid alcohol was read in a colorimeter against the color got through the action of the same kind of alcohol on a chloroform solution of bilirubin (Schuchardt) containing 1 mg. in every 4 cc.¹¹

The early results indicated that not only was the bilirubin output not increased during several successive days of diuresis, but that toward the end of the period it fell almost to zero. Since such a finding is contrary to all that is known of the effects of diuresis on renal elimination, control tests were undertaken.

Various mixtures of icteric and non-icteric urines, some of them concentrated, some dilute as the result of diuresis, showed that the difficulty did not lie with the method itself, which quantitated bilirubin equally well, no matter how dilute the urine.

Further controls, involving variations in the amount of sodium carbonate used in the alkalization of the urine, and in the concentration of acid in the acid alcohol used to redissolve the precipitate, proved that these factors had no essential influence to cause error. Incidentally, it may be remarked that precipitated calcium bilirubinate was found to keep well in the ice box, and the precipitate from our specimens was sometimes kept for 24 to 48 hours prior to quantitation.

At length, tests were made to determine the value of cage as compared with catheterized specimens. The observations indicating a decreased bilirubin output during diuresis had been made upon urine collected in a vessel placed beneath the metabolism cage in which the animal was kept. Such urine was only protected from fecal contamination by a coarse grating in the bottom of the cage. The animal during non-diuresis periods drank very little water and almost invariably passed a dry formed stool, so that during these periods the amount of fecal contamination of the urine was slight. During periods of forced diuresis, on the other hand, a loose watery stool was frequently passed, and the urine specimens then contained large amounts of fecal material. Our practice had been to filter the mixed total urine for the 24 hour period and make tests on the filtered specimen. Parallel tests now made on filtered and unfiltered specimens disclosed considerable differences in bilirubin content (Table II). What is more, it was found that the sediment of a centrifuged cage specimen, though twice washed with distilled water, still may contain as much as four times the amount of bilirubin present in the urine from which it had been separated.

Since the animals serving as our example (Table II) had complete biliary obstruction, as subsequently determined at autopsy, while, furthermore, repeated

¹¹ McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1921, xxxiii, 731.

tests of their stools when unexposed to urine invariably gave negative reactions for bilirubin, the fecal matter can be excluded as the original source of the pigment.

We believe that these findings point to a precipitation of bilirubin out of the urine by some of the fecal elements. They have been set forth at length because it is the accepted practice to use cage urine for bilirubin estimations.

TABLE II.

Effect of Fecal Contamination on the Quantitation of Bilirubin in the Urine.

Dog.	Urine specimen.	Total 24 hr. output of bilirubin as calculated from the filtered and unfiltered specimens.		Remarks.
		Filtered.	Unfiltered.	
		mg.	mg.	
1 Male mongrel; weight 5 kg.	1	19	18	Practically no feces present.
	2	13	13	" " " "
	3	8	23	Marked fecal contamination.
	4	6	14	" " "
	5	11	19	" " "
	6	11	16	" " "
	7	6	13	" " "
2 Male mongrel; weight 6 kg.	1	9	13	Marked fecal contamination.
	2	4	5	" " "
3 Male mongrel; weight 8 kg.	1	5	6	Slight fecal contamination.
	2	9	8	" " "

It has been usually assumed that in the intestinal canal bilirubin is transformed to urobilin and related substances by the action of bacteria. We have observed that when bile-containing urine is mixed with bile-free fecal matter and incubated at 37°C. for 24 hours, a large proportion of its bilirubin is destroyed, while the remainder, no longer in solution, is to be found with the fecal sediment.

Following the recognition of these sources of error, some further carefully controlled experiments were made upon the effects of diuresis in jaundiced dogs.

TABLE III.
Effect on Bilirubin Excretion of Diuresis from Water by Mouth.

Period.	Day.	Duration of excretion.	Urine.					Blood.		Water by gavage.	Remarks.
			Amount.	Output per hr.	Type of specimen.	Bilirubin content.	Bilirubin output per hr.	Hemoglobin.	Bilirubin content per 100 cc. of plasma.		
First non-diuresis period.	1	hrs.	cc.	cc.		mg.	mg.	per cent	mg.	cc.	
		6	72	12.0	Catheterized.	5.61	0.93	69	4.04		
		18	115	6.4	" and voided.	16.56	0.92				
	2	Total or average.	187	7.8		22.17	0.92				Weight 5.2 kg.
		6	35	5.8	Catheterized.	7.10	1.18	69	4.06		
		18	91	5.0	" and voided.	21.20	1.18				
	3	Total or average.	126	5.2		28.30	1.18				
		6	42	7.0	Catheterized.	5.41	0.90	72	4.92		
		18	109	6.1	" and voided.	17.36	0.96				
	4	Total or average.	151	6.3		22.77	0.95				
		6	24	4.0	Catheterized.	4.83	0.80	72	4.78		
		18	70	3.9	" and voided.	15.80	0.88				
		Total or average.	94	3.9		20.63	0.86				

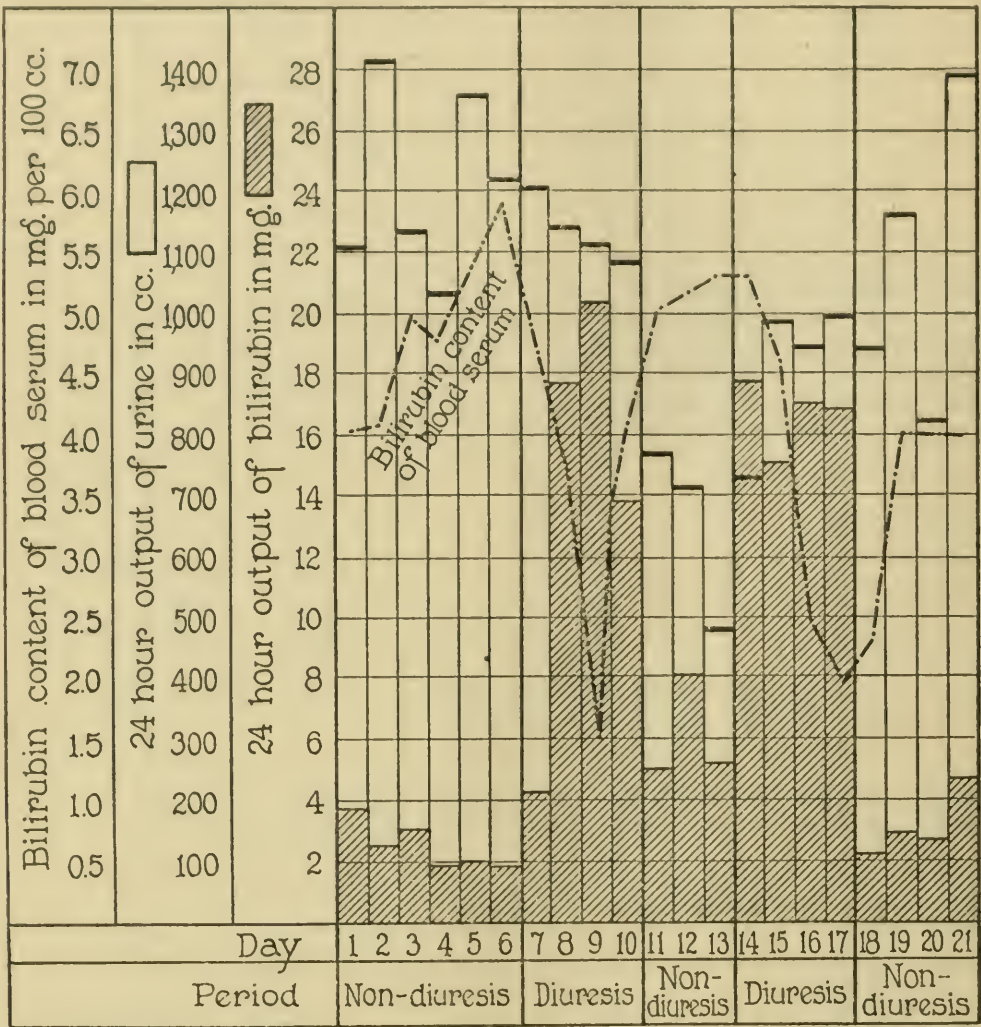
	5	24	100	4.2	Voided.	27.20	1.13			
	6	6	25	4.2	Catheterized.	5.00	0.83			
		18	67	3.7	" and voided.	19.40	1.08	72	5.90	
		Total or average.	92	3.8		24.40	1.02			
First diuresis period.	7	6	33	5.5	Catheterized.	6.43	1.07			500
		18	178	9.9	" and voided.	17.71	0.99			
		Total or average.	211	8.8		24.14	1.00			
	8	6	750	125.0	Catheterized.	7.73	1.29			
		18	133	7.4	" and voided.	15.11	0.84	59	3.7	750
		Total or average.	883	36.8		22.84	0.95			
	9	6	579	96.0	Catheterized.	7.10	1.18			
		18	439	24.4	" and voided.	15.12	0.84	72	1.5	750
		Total or average.	1,018	42.5		22.22	0.93			
	10	6	495	82.5	Catheterized.	6.54	1.09			
		18	195	10.8	" and voided.	15.22	0.85	62	4.04	1,000
		Total or average.	690	28.7		21.76	0.91			Weight 5 kg.

TABLE III—Concluded.

Period.	Day.	Duration of excretion.	Urine.					Blood.		Water by gavage.	Remarks.	
			Amount.	Output per hr.	Type of specimen.	Bilirubin content.		Bilirubin output per hr.	Hemoglobin.			
						mg.	mg.		percent			Bilirubin content per 100 cc. of plasma.
Second non-diuresis period.	11	24	250	10.4	Voided.	15.40	0.64	57	5.03	cc.	Weight 5 kg.	
	12	24	405	16.9	Voided.	14.13	0.59					
	13	24	260	10.8	Voided.	9.60	0.40	56	5.30			
Second diuresis period.	14	24	885	36.8	Voided.	14.60	0.61	56	5.30	750		
	15	6	399	66.5	Catheterized.	6.27	1.04	49	4.60	750		
		18	352	19.5	" and voided.	13.50	0.75					
	16	Total or average.	751	31.3		19.77	0.82					
		6	320	53.4	Catheterized.	6.11	1.02	43	2.45	1,000		
		18	530	29.4	" and voided.	12.76	0.71					
		Total or average.	850	35.4		18.87	0.79					

	17	6 18 Total or average.	688 153 841	114.8 8.5 35.1	Catheterized, " and voided.	6.11 13.79 19.90	1.02 0.77 0.83	43	1.95	800	
Third non-diure- sis period.	18	24	109	4.5	Voided.	18.80	0.78	43	2.3		
	19	24	144	6.0	Voided.	23.20	0.97	45	4.0		
	20	24	132	5.5	Voided.	16.40	0.68				
	21	24	232	9.7	Voided	27.8	1.16	45	4.0		Weight 4.2 kg.

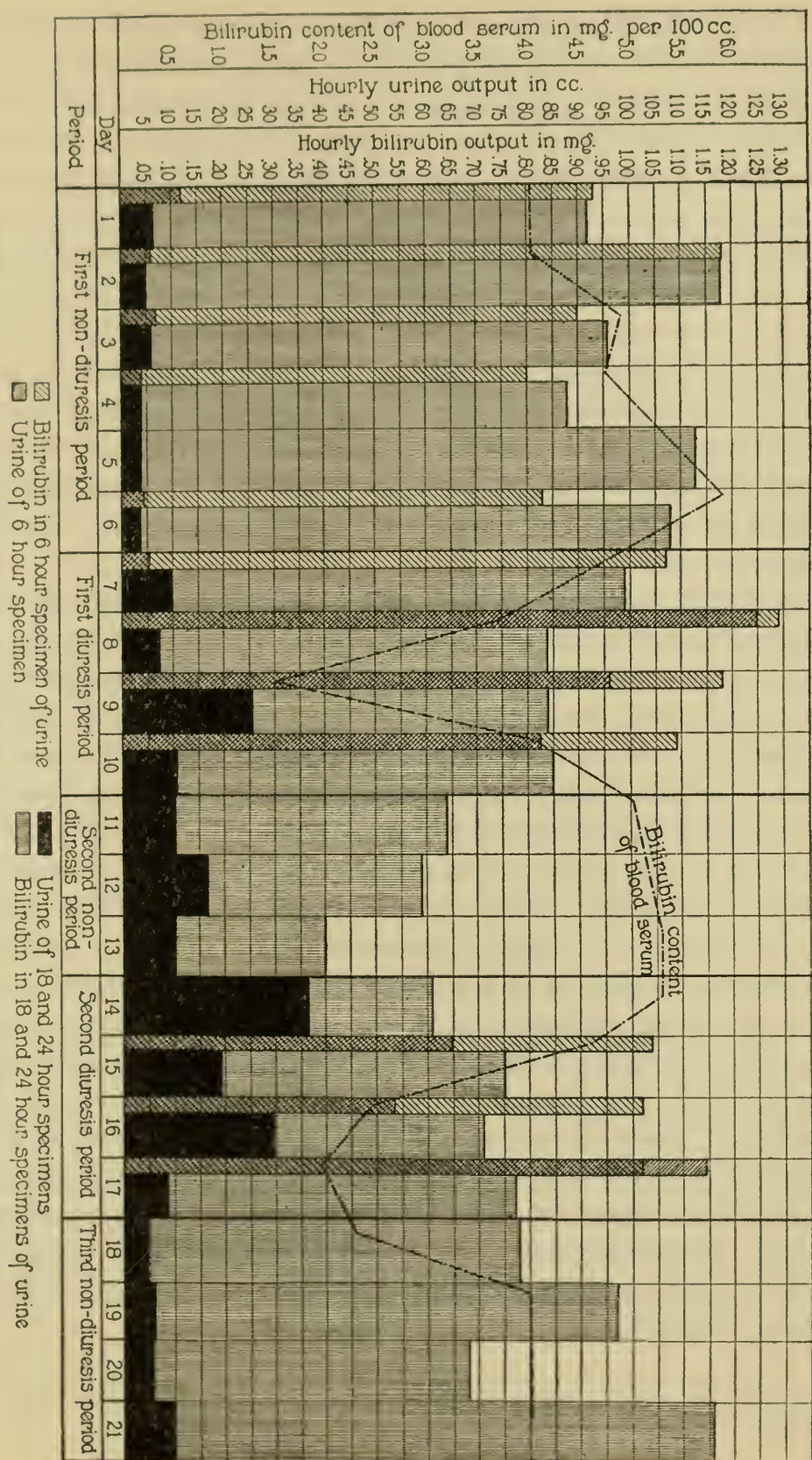
Thymol was added to the urines to check bacterial action; unfiltered specimens were employed, with their proportionate share of fecal sediment when this latter was present; and as much as possible of the 24 hour output was obtained by catheterization. Precipitation with calcium chloride was carried out on each specimen immediately after it had been procured. The bilirubin output was



TEXT-FIG. 2. Effect on bilirubin excretion in the urine of diuresis from water by mouth.

quantitated during alternate periods in one of which no effort was made to increase the water intake, which was invariably low, while in the other water was given by stomach tube, usually in 250 cc. amounts at 2 hour intervals three times each day.

A type instance of the results of the experiments is recorded in Table III and portrayed in Text-figs. 2 and 3. It will be noted that



TEXT-FIG. 3. The same as Text-fig. 2 except that the results are presented in greater detail.

on the 1st day when water was given by stomach tube, practically no diuresis ensued, owing probably to the fact that the animal had drunk but little for some days previously and fluid was needed by the tissues. A similar result was also obtained in later observations on diuresis in jaundiced human beings. On all subsequent days in which water was given there occurred marked diuresis, but as the greater part of the water was eliminated within 2 hours after each administration of it, there was only about 6 hours of diuresis altogether in each 24. The average urinary output for the remaining 18 hours was but slightly greater than that during the control, non-diuresis, periods. Cushny¹² has brought evidence that flood diuresis, such as follows upon intravenous injections, is practically never caused by fluid absorbed from the gastrointestinal canal, and we must assume that it had not occurred in our animals, despite the rapidity of the fluid elimination.

It will be seen that the bilirubin output per hour is fairly regular throughout each day of the first or non-diuresis period, the hourly average between 10 a.m. and 4 p.m. being in general somewhat lower than during the remaining 18 hours. Unfortunately no figures are available for the hourly output between 10 a.m. and 4 p.m. in the second and third non-diuresis periods. Only the average output per hour for the 24 hour period was recorded. In both of the diuresis periods, as already remarked, the increase in quantity of the urine was limited practically to the period between 10 a.m. and 4 p.m. The hourly output of bilirubin during this time is distinctly greater than during the remainder of the day. On the other hand, the output in the remaining 18 hours, when the urine was relatively scanty, is decreased to such extent that the output for the entire 24 hours averaged a little less than that during the first and third non-diuresis periods. True, the average 24 hour output for the second non-diuresis period is the lowest of the series, but there is no evidence to show that this was due to the administration of water during the preceding days. The main point sought after is clearly shown; diuresis from water by mouth has remarkably little effect on the day to day elimination of bilirubin.

¹² Cushny, A. R., *The secretion of the urine*, London, New York, Bombay, Calcutta, and Madras, 1917, 136.

The bilirubin of the blood plasma was followed by means of the diazo reaction, as has already been stated. There occurred a fall in the pigment concentration during the diuresis periods, but this can be explained in several ways other than by increased elimination of bilirubin from the body, notably by the accumulation of fluid in the tissues. Both in dogs and in human beings a fluid retention was indicated by changes in the weight. Quite possibly less bile pigment was produced in the organism during the periods of diuresis than in the intervals between.

Effects of Diuresis in Jaundiced Patients.

Observations were made on two men with catarrhal jaundice in whom periods of diuresis from forced fluid by mouth were alternated with periods of restricted water intake.¹³ The icterus was marked when the observations were begun, but thereafter gradually and regularly diminished. The marked variations that were induced in the daily output of urine through forcing water by mouth had no evident effect on the rate at which the jaundice lessened from day to day as determined by the diazo reaction on the blood plasma. And the content of the 24 hour urine in bilirubin was no greater when the voidings amounted to several liters than when only half of this quantity had been passed. The findings, then, confirming those in dogs, show that diuresis by alimentary means fails, practically speaking, to increase bilirubin elimination.

DISCUSSION.

The foregoing experiments offer little support for the view advanced by some clinicians that forcing water by mouth has a direct effect to diminish the intensity of jaundice, by increasing the elimination of bilirubin in the urine. The flood diuresis which follows an intravenous injection of salt solution brings out, it is true, a relatively considerable quantity of bile pigment, but the method is clinically inapplicable, and, as has been shown, a copious diuresis from water, by mouth, yields no such consequence, so far as the 24 hour output is concerned. The fact is well recognized that "all the constituents

¹³ These observations were made through the courtesy of Dr. E. F. Du Bois.

of the urine are increased in absolute amount per unit of time during diuresis."¹² In the present instance, the increase is more important theoretically than actually. Yet obviously this need not mean that diuresis is valueless as a means wherewith to combat the effects of bile retention. Its influence on the output of bile salts—substances more injurious to the organism than the pigments—remains to be determined when proper methods become available. Furthermore, diuresis may help to avert the accumulation of bilirubin in the kidneys with the disordered function consequent thereon.

Nonnenbruch¹⁴ has shown that the acutely disordered kidney may fail to eliminate bile pigment during jaundice. It is probable that the extraordinarily pronounced icterus seen in some human beings during the later weeks of total biliary obstruction is due in part at least to a lessening of renal elimination. We have obtained data which would seem to bear significantly upon this point. In the attempt to increase jaundice in dogs, some injections of hemoglobin were given intravenously to animals already the subject of a long standing, total biliary obstruction and the mild general icterus that this entails. Hemoglobin for the purpose was obtained by the method of Sellards and Minot;¹⁵ and eight to ten successive hourly injections were given, of amounts slightly less than that which should, on calculation, cause hemoglobinuria. In this way it proved possible to intensify the icterus markedly. At the end of a day of injections, the scleras of the dogs were pronouncedly more yellow than at the beginning. But the icterus was not maintained. By next morning only the previous, relatively pale, coloration characteristic of total obstruction was present. The urine during the transition period was heavily loaded with bilirubin. It is probable that in the case of bilirubin, as of urea, any increase in the circulating amount beyond a certain point is compensated for, when the kidneys are normal, by an increase in ease of elimination.

In man, as already stated, bilirubin is a threshold substance, in its renal relations, and it is normally present in appreciable amount in the blood. But in the dog, not only is the blood normally free from it, but whenever bilirubinemia can be detected there is bilirubinuria,

¹⁴ Nonnenbruch, W., *Mitt. Grenzgeb. Med. u. Chir.*, 1918-19, xxxi, 470.

¹⁵ Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917-18, xxxvii, 161.

while often the latter is to be found alone.¹¹ The question arises whether actually there is no threshold for bilirubin in this species, or whether the current tests for bilirubinemia are at fault. The presence in freshly voided urine, during even the slightest bilirubinuria, of renal cells specifically stained or stippled with the bile pigment constitutes evidence in this connection. According to the modern view enunciated by Cushny, all substances that pass into the urine leave the circulation by way of the glomerulus, and the so called threshold substances undergo resorption to a greater or less degree during their passage through the tubules. Thus it is that when the threshold substance is a dyestuff, the tubular epithelium becomes pigmented. Were all this quite certain, the existence in the urine of the dog of jaundiced kidney cells would be proof that in

TABLE IV.
Maximal Bilirubin Output during Total Obstruction.

Dog No.	Body weight.	Bilirubin.		Time of occurrence after operation.
		Expected 24 hr. output.	Greatest actual 24 hr. output.	
	<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	<i>days</i>
1	4½	39.6	19.5	28
2	5½	45.8	28.3	28
3	9¼	81.4	24.0	10

this species bile pigment is a threshold substance. But as happens, there is recent work to show that some, at least, of the tubules have an excretory function.¹⁶ Therefore, our cellular evidence is unconvincing.

The amount of bile pigment eliminated in the urine of the dog during complete and long continued biliary obstruction is never nearly so great as that formed during the same period by a normal liver. The bilirubin output of fistula animals in good condition amounts to about 8.8 mg. per kilo of body weight per day.⁷ Table IV shows the observed pigment content of the urine of three dogs with persistent total obstruction. It will be seen that the maximum output of Dog 3 occurred on the 10th day of obstruction and that

¹⁶ Oliver, J., *J. Exp. Med.*, 1921, xxxiii, 177.

of the other two animals on the 28th day. In all three instances the bilirubin content of the blood had already become fairly constant, and so too had the tissue icterus. The 24 hour output of pigment in the urine never approached the amount which a normal liver would have secreted in the same time. Several explanations suggest themselves for the discrepancy, more especially diminished liver function during jaundice, and destruction of pigment within the body.

SUMMARY.

The elimination of bile pigment during jaundice is, for practical purposes, unincreased by diuresis from water by mouth. Possibly, though, the flushing of the kidneys tends to lessen pigment accumulation within these organs and thus to diminish a serious potential source of trouble in long continued jaundice. Flood diuresis from intravenous injections of salt solution markedly increases the output of bile pigment. It is important to know the effect of variations in the urinary output on the elimination of bile salts, but methods for the purpose are not available at present.

The passage of bile pigment into the kidney cells during jaundice is attested by the presence in the freshly voided urine of desquamated renal elements specifically stained, stippled, or granulated with bilirubin. Pigmentation of this sort is readily to be distinguished from the indiscriminate staining of cellular debris that occurs in icteric urines on standing. It has clinical significance, furnishing direct evidence on the degree of renal change.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VII. SEROLOGICAL REACTIONS.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 37.

(Received for publication, December 10, 1921.)

During the fall and winter of 1918-19, and the early spring of 1920, we collected a number of specimens of blood serum from patients in the active stages of epidemic influenza, or after recovery from the disease, for the purpose of studying the reactions of the sera with strains of *Bacterium pneumosintes*¹ which had been isolated from the nasopharyngeal secretions of influenza patients and from the lung tissues of rabbits inoculated with these secretions. Samples of blood serum were collected also from rabbits which had been allowed to recover after showing the characteristic clinical picture produced by intratracheal injections of the active nasopharyngeal secretions, or had been experimentally inoculated with the sediment from tissue cultures of *Bacterium pneumosintes*.

The results of our efforts to demonstrate specific antibodies in these serum specimens were disappointing. The sparse growths of the organism in the earlier generations, mixed with the protein precipitate that develops in the Smith-Noguchi medium, did not provide an antigen suitable for serological tests. On account of non-specific precipitation and concomitant sedimentation, precipitin and agglutinin tests were unsatisfactory and indeterminate. Therefore, at the time the sera of influenza patients and of most of the affected rabbits were available we were unable to make use of them for lack of a suitable antigen.

¹ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

More recently a method has been developed by one of us (Gates) by which certain pathogenic anaerobes, including *Bacterium pneumosintes*, may be cultivated in a collodion sac dialysate of the Smith-Noguchi medium.² The ascitic fluid or dilute serum and the fresh tissue fragment are placed within the sac, which is surrounded by distilled water or physiological salt solution under a vaseline seal. In the course of 24 to 48 hours anaerobic conditions are established throughout the system and the nutritive and growth-promoting substances in the tissue medium have diffused through the membrane in sufficient quantities to support growth in the surrounding liquid, while the protein precipitate that collects around the tissue fragment is retained within the sac.

Bacterium pneumosintes grows readily in this anaerobic tissue culture dialysate, visibly clouding the clear liquid in a few hours and producing a heavy turbidity in 3 to 5 days. When growth is checked through exhaustion of the nutritive material or the accumulation of deleterious substances the organisms gradually settle out of suspension, leaving a clear supernatant fluid over a compact, slightly brownish sediment. Films of these cultures show only the stained organisms, without the background of precipitate that is deposited by the tissue medium.

Preparation of Immune Serum.

When it was possible to cultivate *Bacterium pneumosintes* by this method in quantities sufficient for use, two rabbits were injected intravenously with doses of the living culture of Strain 11. This strain had been recovered from the lung tissue of a rabbit representing the eighth animal passage of active material derived from the nasopharyngeal secretions of Patient 11³ of the 1918-19 epidemic. During immunization one rabbit (A) developed secondary infections and was killed. The other rabbit (B) received five injections of 2 to 4 cc. of a thin suspension of live culture at intervals of 5 to 7 days and was bled on the 9th day thereafter. The sterile serum was stored at 4°C. without preservative. In the first series of tests with

² Gates, F. L., *J. Exp. Med.*, 1922, xxxv (in press).

³ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

this serum it was examined for the presence of agglutinins, precipitins, bacteriotropins, and complement-fixing bodies against two strains of *Bacterium pneumosintes*, No. 11, the homologous strain, and No. 26, from the recurrent epidemic of 1920.

Since then the serum of this rabbit and similar sera produced in five other rabbits have been studied in various serological reactions with four strains of *Bacterium pneumosintes*, three (Nos. 11, 16, and 17) from the first epidemic (1918-19), and one (No. 26) from the second (1920). The results of the latter experiments are all in accord with those of the first series and need not be reported in detail. The following tests are described to indicate the methods and results of the first serological experiments.

Agglutination.

Agglutination tests were first made with a mixture of live organisms and undiluted immune serum from Rabbit B spread on a cover-slip, sealed with vaseline over a hollow slide, and examined microscopically immediately or after 30 minutes incubation at 37°C. Both strains tested (Nos. 11 and 26) were promptly agglutinated, with the formation of large compact masses of organisms in a clear surrounding fluid (Fig. 1). Control tests with normal rabbit serum showed no agglutination, or only the occasional clumping of three or four bacteria.

Macroscopic agglutination tests were then set up to determine the titer of the immune serum. Measured drops of serum dilutions 1:5 to 1:160 were mixed with equal quantities of bacterial suspension and sealed in capillary tubes of 2 mm. internal diameter.⁴ After incubation for 2 hours at 37°C. the tubes were placed in the ice box over night and read the next morning. Strain 11 was completely agglutinated in a serum dilution of 1:80, with the limit of visible agglutination in a serum dilution of 1:320. The corresponding limits for Strain 26 were 1:40 and 1:160, respectively. Control tests with normal rabbit serum showed no agglutination even in a serum dilution of 1:2. The contrast in the behavior of Strain 11 in immune and in normal serum is shown in Fig. 2.

Precipitation.

Precipitin tests were also performed in glass tubes of 2 mm. bore, in which the antigen and the serum were carefully layered by drawing them up in succession by means of a rubber bulb. The antigen used was the clear supernatant fluid from old cultures of *Bacterium pneumosintes* grown in the dialysate of a tissue medium. After incubation for 1 hour at 37°C. a sharp opaque line of precipitation

⁴ Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvii, 2054.

was observed at the juncture of the antigen and the immune serum from Rabbit B. In undiluted, normal rabbit serum a doubtful line of precipitation was observed with Strain 11 only. The appearance of the experimental and control tubes is shown in Fig. 3.

Complement Fixation.

The antigens used in the complement fixation test were the same as those for the precipitation reaction. Preliminary control tests showed that these antigens were not hemolytic or anticomplementary in volumes of 0.5 cc. when tested with 0.1 cc. of 40 per cent guinea pig complement and 2 units of anti-human amboceptor against 0.1 cc. of a 10 per cent suspension of human red cells. The immune serum of Rabbit B and the control normal serum were not hemolytic or anticomplementary in dilutions of 1:5. Antigen 0.45 cc., complement 0.1 cc., and the test sera in dilutions up to 1:160, made up to a volume of 1.05 cc. with physiological saline solution, were incubated together in a water bath at 37°C. for 30 minutes. After the addition of 0.1 cc. of corpuscle suspension and 0.1 cc. (2 units) of dilute amboceptor the tubes were again incubated for 30 minutes at 37°C. Complement fixation was complete with both antigens (Strains 11 and 26) in the highest dilution of immune serum tested; namely, 1:160. No fixation occurred in the tubes containing the normal rabbit serum.

Phagocytosis.

In the phagocytic experiments the Neufeld method⁵ was used to test for the presence of bacteriotropic substances. Rabbit leucocytes were obtained from subcutaneous tubes⁶ containing aleuronat in agar. Large mononuclear cells (monocytes) and lymphocytes, as well as polynuclear neutrophils, collect in these tubes and may be observed in phagocytic experiments. In the tests to be described the diluted leucocytic suspensions were mixed with suspensions of young dialysate cultures of *Bacterium pneumosintes* in physiological salt solution, or in dilutions of normal or immune rabbit serum. The mixtures were incubated 1 to 4 hours at 37°C., films prepared, fixed in methyl alcohol or by heat, and stained with Löffler's alkaline methylene blue, Wright's blood stain, or Cross' stain for leucocytes.⁷ In salt solution or in normal serum controls only an occasional leucocyte picked up a few organisms. Unphagocytosed bacteria were plentiful and unagglutinated. In the presence of immune serum from Rabbit B, however, two phenomena occurred. Especially in low dilutions of the immune serum the bacteria were gathered into clumps. Leucocytes had attacked and

⁵ Neufeld, F., and Rimpau, W., *Deutsch. med. Woch.*, 1904, xxx, 1458; *Centr. Bakt., Ite Abt., Ref.*, 1906, xxxvii, 763.

⁶ Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, xviii, 280.

⁷ Cross, H. B., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 51.

sometimes surrounded these clumps, with which they were engorged. Other leucocytes had engulfed single organisms and contained from a few to very many of them. Both the polynuclear cells and the large mononuclear cells (monocytes) were actively phagocytic. Small mononuclear cells (lymphocytes) did not engulf the bacteria. The highest serum dilution in which an increased phagocytic activity was clearly evident was 1:80. Photographs of these phases of the reaction are shown in Figs. 4 to 7.

In addition to this series of experiments with the serum of Rabbit B and Strains 11 and 26 of *Bacterium pneumosintes*, we have made serological tests with Strains 16 and 17 and with the serum of five other rabbits immunized by repeated injections of these strains. Sera produced with certain strains, when tested in cross-agglutination experiments with the other strains, have agglutinated them and the homologous organisms in practically the same dilutions. No specific differences among the three strains from the 1918-19 epidemic or between any of these and Strain 26 from the recurrent epidemic of 1920 have been found, and the serological evidence indicates their antigenic identity. This is what might be expected if they were all derived from a common source.

It is evident from the results of these experiments that the reaction of the animal body to *Bacterium pneumosintes* involves the production of the antibodies which are commonly recognized by serological tests. We have no reason to suppose, therefore, that the mechanism of protection against this organism, of which we have evidence in the immunity reactions already described,⁸ differs from that which comes into play in the case of infections with aerobic pathogenic organisms.

We would have welcomed the opportunity of testing the sera of influenza patients during the recent epidemic, or of rabbits experimentally infected with nasopharyngeal secretions from these patients. At first a suitable antigen was lacking. More recently almost all of the glycerolated material on hand was rendered inactive through the inadvertent use of glycerol which was later found to be chemically impure. The following single experiment, therefore, represents our only opportunity to test against *Bacterium pneumosintes* the sera of rabbits which had been subjected to the active agent in glycerolated form.

⁸ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

Specific Agglutinins in the Blood of Rabbits after the Injection of Glycerolated Material.

September 12, 1921. Two rabbits were injected intratracheally with glycerolated lung tissue from a previously injected rabbit which had showed the typical clinical and pathological picture already described.³ This animal represented the seventh passage of the active agent derived from Case 26.³ The two injected rabbits in turn showed conjunctivitis, an increase in temperature, and the characteristic fall in the total leucocyte count due to a drop in mononuclear cells. Both were allowed to recover, and were bled from the ear vein for a serum sample

Serum dilutions														
Serum	Strain	1:2	1:4	1:8	1:16	1:32		Serum	Strain	1:2	1:4	1:8	1:16	1:32
Immune rabbit B	16	++	++	++	++	++		Normal rabbit 3	16	-	-	-	-	-
	17	++	++	++	++	++			17	-	-	-	-	-
	26	++	++	++	++	++			26	-	-	-	-	-
Experimental rabbit 1	16	++	+	+	-	-		Normal rabbit 4	16	-	-	-	-	-
	17	++	+	+	-	-			17	-	-	-	-	-
	26	++	+	-	-	-			26	-	-	-	-	-
Experimental rabbit 2	16	++	+	-	-	-		Normal rabbit 5	16	-	-	-	-	-
	17	++	+	+	-	-			17	-	-	-	-	-
	26	+	+	-	-	-			26	-	-	-	-	-

TEXT-FIG. 1. Agglutinins in the serum of rabbits injected with active glycerolated lung tissue.

on the 21st day after injection. These sera together with three normal rabbit sera and the immune serum from Rabbit B were then tested for agglutinins against killed, washed, dialysate cultures of *Bacterium pneumosintes* (Strains 16, 17, and 26). The results of this experiment are shown in Text-fig. 1.

This experiment shows that the animal body reacts to experimental infection with the active agent of the nasopharyngeal secretions of influenza patients by antibody formation against *Bacterium pneumosintes*, and completes the proof of the identity of the active agent with the organism obtained from the same immediate and original sources.

SUMMARY.

Cultivation of *Bacterium pneumosintes* in the collodion sac dialysate of a tissue medium produces an antigen suitable for serological tests.

Injection of dialysate cultures of *Bacterium pneumosintes* into rabbits results in the production of antibodies demonstrable by agglutination, precipitation, complement fixation, and phagocytic reactions.

Four strains of *Bacterium pneumosintes*, three from the first epidemic influenzal wave (1918-19) and one from the second (1920), show identical antigenic characters.

The blood serum of rabbits experimentally injected with the glycerolated active material of rabbit passages contains specific agglutinins for *Bacterium pneumosintes*, whereas normal rabbit serum does not.

EXPLANATION OF PLATE 37.

FIGS. 1 and 2. Agglutination of *Bacterium pneumosintes* in immune serum from Rabbit B.

FIG. 1. Edge of hanging drop, dried and stained. $\times 15$.

FIG. 2. Macroscopic agglutination in capillary tubes. *A*, immune serum, complete agglutination; *B*, normal rabbit serum, no agglutination. The white crescent at the bottom of each column is not sediment but light reflected from the meniscus. $\times 1$.

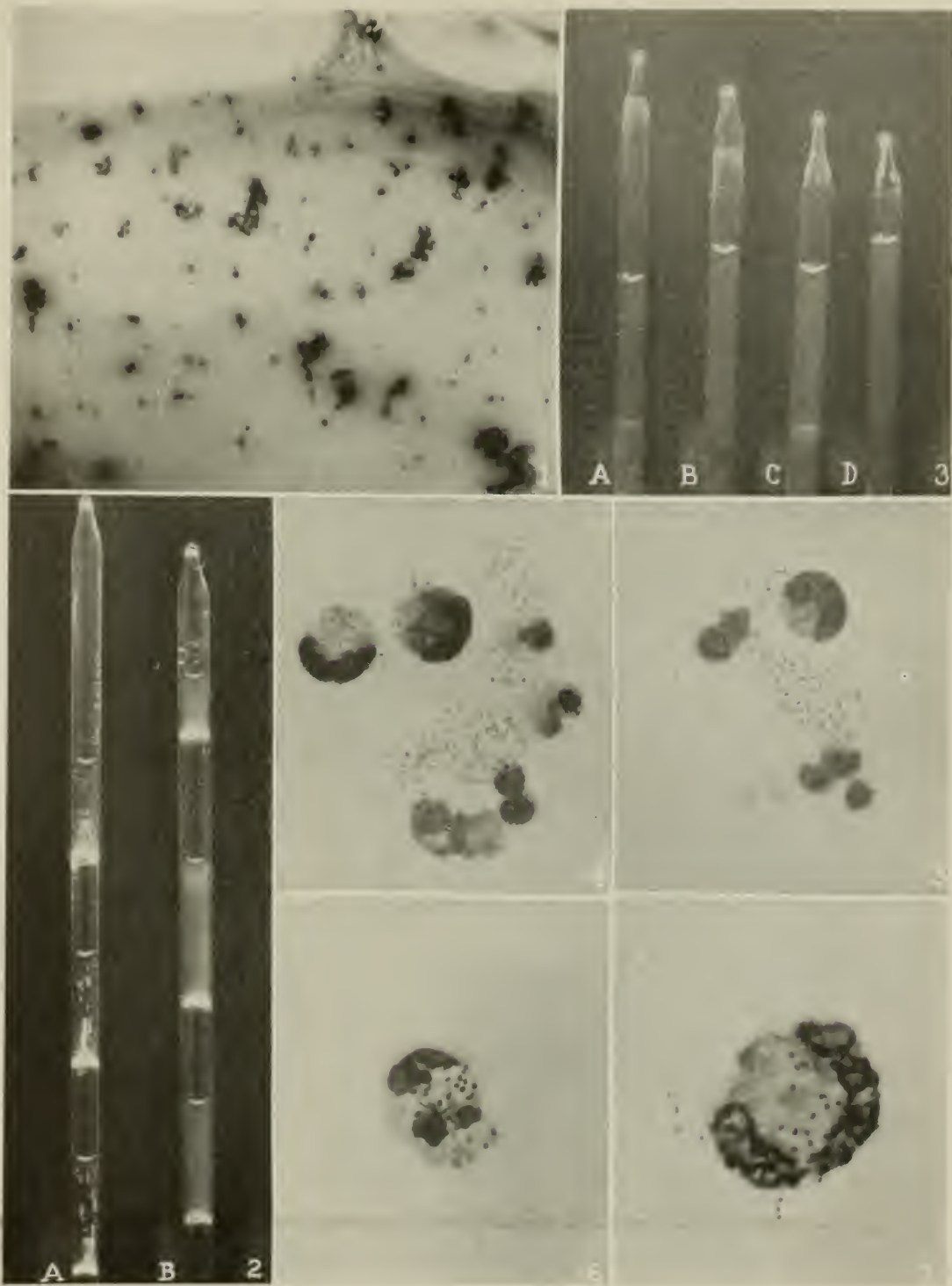
FIG. 3. Precipitation reaction with the supernatant fluid of *Bacterium pneumosintes* cultures versus rabbit serum. *A*, Strain 11 and immune serum from Rabbit B; *B*, Strain 11 and normal rabbit serum; *C*, Strain 26 and immune serum from Rabbit B; *D*, Strain 26 and normal rabbit serum. $\times 1$.

FIGS. 4 to 7. Phagocytosis of *Bacterium pneumosintes* in immune serum from Rabbit B.

FIGS. 4 and 5. Monocytes (transitional cells) attacking agglutinated groups of organisms. $\times 1,000$.

FIG. 6. Polynuclear neutrophil containing organisms. $\times 1,100$.

FIG. 7. Monocyte containing organisms. $\times 1,500$.



(Olitsky and Gates: Nasopharyngeal secretions from influenza. VII.)

MUTATION OF THE BACILLUS OF RABBIT SEPTICEMIA.

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PLATE 38.

(Received for publication, December 7, 1921.)

INTRODUCTION.

Two varieties of microbe have been shown to exist in cultures of the rabbit septicemia bacillus (1). These organisms have been designated as Types D and G. Type D is the microbe invariably obtained at necropsy of rabbits dying from natural infection. Type G appears after artificial culture has been carried out for some time. It was important to determine whether the two varieties coexist in cultures isolated from infected rabbits, or whether Type G arises by mutation from Microbe D. Much of the work on mutation of bacteria has been criticized because of failure to employ pure-line strains, that is to say, cultures arising from single organisms. Since the primary isolations of Microbe D were made from colonies which conceivably might arise from two or more organisms, it would be unjustifiable to conclude that the original Type D had changed into the microbe of the G variety. Consequently, the experiments about to be reported were performed with pure-line strains isolated from a Type D culture by the Barber method (2).

Differential Characteristics of Microbes D and G.

These have been given in detail in a preceding communication (1) but will be briefly summarized here.

Type D is very virulent for rabbits, grows diffusely in liquid media, and yields smooth *fluorescent* colonies on serum agar. The acid agglutination optimum lies between pH 3.5 and pH 3.0 (Michaelis-Beniasch buffer series (3)).

Type G is of very low virulence for rabbits, exhibits granular growth in fluid media, and grows in the form of slightly irregular, translucent, *non-fluorescing*

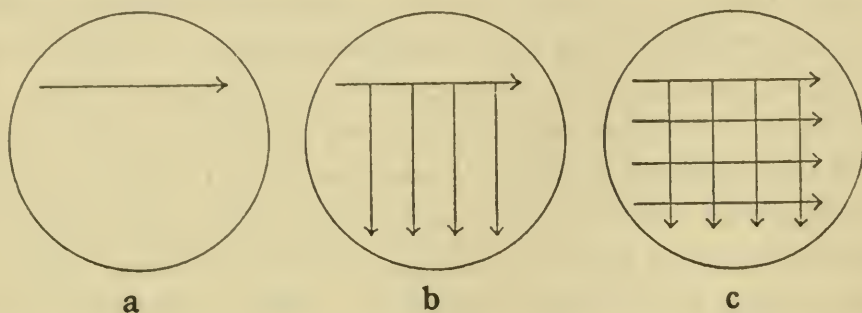
colonies on serum agar. Its acid agglutination optimum lies between pH 5.0 and pH 3.5.

There is no noticeable morphological difference between the two varieties. Their fermentation reactions are indistinguishable. Immunization and agglutination absorption experiments indicate that they are antigenically homologous.

Method of Detection of Microbe G in Cultures in Which Microbe D Predominates.

This is made comparatively easy by the striking difference in appearance of the colonies on streaked serum agar plates, especially when the latter are held at certain angles before artificial light.

The tube of liquid medium supposed to contain the mixture of Types D and G is thoroughly shaken in order to bring into suspension clumps of Type G which may possibly have sedimented. A large



TEXT-FIG. 1, *a* to *c*. Diagrammatic sketch of the method of streaking serum agar plates.

platinum loop of the material is then streaked over the surface of 10 per cent rabbit serum agar in Petri dishes of 15 cm. diameter.

The loop is then sterilized, and successive streaks are made at right angles to the first one and starting from it. Then, without sterilizing the loop, it is passed across these second streaks and parallel to the original one. The resulting growth will therefore have a checker-board appearance. The growth is heavy in the upper left-hand corner, the point where the loop was first applied. It will be much scantier in the lower right-hand corner. The successive stages of the operation are shown in the diagrammatic sketches in Text-fig. 1, *a* to *c*.

The result may be seen in Fig. 1. In this figure the Type D colonies can be readily detected, though in the minority. They are white while the Type G colonies appear gray.

The comparative scantiness of the colonies in the lower latitudinal streaks makes it possible to make rough quantitative estimates of the relative proportion of the Type D and G colonies.

Isolation of Pure-Line Strains.

The rabbit septicemia bacillus is rather exacting in its nutritive requirements, and it is necessary to make fairly generous seedings during routine transplant. As a result, difficulty was at first experienced in obtaining positive cultures by the Barber method. This was overcome by performing the entire operation in undiluted rabbit serum. The cultures used for fishing had been incubated in undiluted rabbit serum for 3 hours. Minute drops of these young cultures were placed in the usual manner in the Barber cell, examined by oil immersion lens, and when found to contain single organisms, fished to undiluted rabbit serum, and incubated. The percentage of positive cultures obtained by this method is relatively high. On the other hand, in plain or serum broth the results are very unsatisfactory.

EXPERIMENTAL.

The following observation led to the experiments about to be presented. Several strains of Microbe D, isolated at necropsy from rabbits dead of bronchopneumonia, were carefully purified as follows: A 6 hour serum broth culture was diluted in broth to 10^{-8} cc. of the original. Plates in serum agar made from this dilution usually showed from two to five colonies. One of these was fished to serum broth, and as soon as a cloud appeared (about 6 hours) the dilutions were again made and 10^{-8} cc. plated as before. This process was repeated four times in all. The young cultures used for these dilutions showed few or no clumps. The high dilutions— 10^{-8} , 10^{-7} , and 10^{-6} cc.—when plated, yielded numbers of colonies that increased in direct proportion with the lowering of the dilution. That is to say, 10^{-8} plate, 2 colonies; 10^{-7} plate, 19 colonies; 10^{-6} plate, 205 colonies. These considerations make it certain that at some, and probably at each time during the fishing process a colony was obtained arising from *one* organism.

The Type D strains so purified were now subcultured daily in undiluted rabbit serum and in plain broth. Each culture was streaked

on 10 per cent rabbit serum agar in the manner just described. Careful inspection for Type G colonies was made. From the twentieth to the twenty-fifth passage, these began to make their appearance in small numbers in the plain broth series. None could be demonstrated in the plates made from undiluted serum.

One tube of the plain broth series which had shown *no* Type G colonies was allowed inadvertently to stand for 4 days at room temperature. It was then streaked, and large numbers of Type G forms appeared on the serum agar plate. None were present in a 4 day serum culture of the same Type D strain. The Type G colonies, subcultured from the plain broth plate, bred true to type and showed all of the characteristics previously described. It was concluded that an undoubted mutation of the original Type D strain had occurred. Eight pure-line strains were then obtained by the Barber method and the following experiments made, to determine, if possible, the conditions favoring and inhibiting the $D \rightarrow G$ transformation.

Experiment 1. $D \rightarrow G$ Mutation in Plain Broth, Serum Broth, and Undiluted Rabbit Serum.—0.05 cc. of a 6 hour undiluted serum culture of Microbe D, pure-line, was inoculated into three large test-tubes containing 10 cc. respectively of undiluted rabbit serum, 5 per cent rabbit serum broth, and plain broth. The tubes were placed in the incubator at 37°C., and streaked on serum agar plates by the method just described, at intervals covering a period of 109 hours. The plates were incubated for 24 hours and then inspected for the appearance of Type G colonies. The serum culture used for inoculation was streaked and observed to contain only Type D. The result of the experiment is given in Table I.

The result of Experiment 1 indicates that the ageing of a culture plain broth causes Microbe D to change into Type G. The addition of a small amount of serum does not greatly inhibit this change. On the other hand, undiluted rabbit serum has a very marked inhibitory effect.

All of the pure-line strains under study have been found to undergo this mutation when allowed to age in plain broth, but do so with varying rapidity and completeness. The Type G colonies, fished into plain broth or undiluted serum, remained true to type indefinitely. They showed no tendency to revert to the parent D form even when transplanted continuously in undiluted rabbit serum, a medium which is antagonistic to the original change.

It was thought that it might be possible to demonstrate mutation-enhancing properties in plain broth cultures of Microbe D. The following experiment examines this question.

Experiment 2. Effect of Type D Culture Filtrates on the Mutation Rate of Microbe D.—A pure-line strain of Microbe D was planted into a series of flasks containing plain broth (pH 7.4). These were incubated at 37°C. After 6, 24, 48, and 72 hours, a flask was removed, centrifuged, and filtered through small Berkefeld candles. The filtrates upon test proved to be sterile. A slight change in reaction had occurred, the 6 and 24 hour filtrates giving pH 7.1, the 48 hour pH 7.0, and the 72 hour pH 6.9. The filtrates were now divided into two

TABLE I.

D → G Mutation in Plain Broth, Serum Broth, and Undiluted Rabbit Serum.

Strain used for inoculation.	Medium.	Proportion of Types D and G.							
		9 hrs.	24 hrs.	36 hrs.	48 hrs.	60 hrs.	72 hrs.	85 hrs.	109 hrs
0.05 cc. of 6 hr. serum culture; pure-line strain; Type D.	Undiluted rabbit serum.	D	D	D	D	D	D	D	D
	5 per cent serum broth.	"	"	"	"	"	" +2G.	" + sev- eral G.	" + G (about 70:30).
	Plain broth.	"	"	"	" + few G.	" + few G.	D + mod- erate number of G.	D + many G.	D + G (about 50:50).

parts, half was titrated back to pH 7.4 with 0.1 N NaOH, the remainder was left without treatment. 10 cc. of each filtrate were placed in large test-tubes. These tubes were now seeded with 0.05 cc. of serum culture of pure-line strain, Type D. Controls, consisting of similar amounts of sterile broth incubated for 6 and for 72 hours, and of undiluted rabbit serum, were similarly seeded. All of the tubes were now incubated at 37°C., and streaks made on serum agar plates at intervals up to 7 days, 10 hours. Growth occurred in all of the filtrates, although a considerable lag period was evident in those of 48 and 72 hours. The results of the experiment are presented in Table II.

TABLE II.
Effect of Type D Filtrates on the Mutation Rate of Microbe D.

Tube No.	Medium.	pH	Proportion of Types D and G.									
			12 hrs.	28 hrs.	43 hrs.	60 hrs.	72 hrs.	84 hrs.	106 hrs.	120 hrs.	138 hrs.	176 hrs.
1	6 hr. filtrate.	7.1	D	D	D	D	D	D + 1 G.	D	D	D	D
2	6 "	7.4	"	"	"	"	"	D	"	D + 2 G.	D + few G.	"
3	24 "	7.1	"	"	"	"	"	"	"	" + few G.	D	D + few G.
4	24 "	7.4	"	"	"	"	+2 G	+ 1 G.	"	" + "	"	D
5	48 "	7.0	"	"	"	"	"	"	D + 1 G.	D + moderate number of G.	D + few G.	D + few G.
6	48 "	7.4	"	"	"	"	"	"	D	D + moderate number of G.	" + "	" + "
7	72 "	6.9	"	"	"	"	"	"	D + moderate number of G.	D + G (50:50).	D + G (50:50).	D + G (50:50).
8	72 "	7.4	"	"	"	"	"	+ 1 G.	D + moderate number of G.	D + G (50:50).	D + G (50:50).	D + G (50:50).
9	Sterile broth (37°C. 6 hrs.).	7.4	"	"	"	"	"	"	D + many G.	D + G (40:60).	D + G (40:60).	D + G (40:60).
10	Sterile broth (37°C. 72 hrs.).	7.4	"	"	"	"	"	+ 12 G.	" + "	D + G (30:70).	D + G (30:70).	D + G (50:50).
11	Undiluted serum.	7.4	"	"	"	"	"	+ 6 G.	D	D	D	D

A glance at Table II shows that the expectation of the discovery of conditions enhancing mutation was not verified. In fact, the opposite property is seen to be present in the 6, 24, and 48 hour filtrates. Contrary to expectation, the number of Type G colonies arising in the 6 and 24 hour filtrates was extremely small, and comparatively few appeared in that of 48 hours. In the 72 hour filtrate the mutation rate paralleled that of the control broth. The mutation had reached 50 per cent or more in 176 hours. In undiluted rabbit serum no Type G colonies appeared at any time during the experiment. Early filtrates from Type D cultures exhibit a markedly antagonistic action to the mutation. Little or no difference is to be observed between the 72 hour filtrate of reaction pH 6.9 and its companion tube, which had been titrated back to pH 7.4. It would appear that the slight change in reaction has no effect upon the mutation. Many of the Type G colonies were subjected to the differential tests, and proved in all cases to be authentic Type G cultures. They did not revert to Type D.

An effort was now made to discover, if possible, the constituents of plain broth that encourage the $D \rightarrow G$ mutation. Plain beef infusion was the first of these elements subjected to test. The beef infusion was prepared in exactly the same way as in the preparation of plain broth. It was titrated to pH 7.4. The only difference between it and the plain broth ordinarily used was the absence of peptone (Fairchild's) and of Na_2HPO_4 , which is added for buffer purposes.

Experiment 3. $D \rightarrow G$ Mutation in Beef Infusion and Other Media.—0.05 cc. of a serum culture of a pure-line strain, Type D, was seeded into large test-tubes containing respectively 10 cc. of 6 hour Type D filtrate, of plain broth, of 5 per cent rabbit serum broth, of beef infusion, of 5 per cent rabbit serum-beef infusion, and of undiluted rabbit serum. The material used for seeding was streaked on serum agar plates and proved to contain only Type D microbes. The plain broth was made up from the beef infusion used in the experiment. All of the tubes were placed at 37°C. in the incubator and tested by streaking on serum agar plates at intervals up to 18 days. The results are given in Table III.

Table III indicates that beef infusion is very unsuitable to the $D \rightarrow G$ change. In 228 hours, frequent streaking of 10 per cent serum agar plates revealed only one Type G colony in this medium. In 18 days

a considerable mutation had taken place. The process, therefore, can take place in this medium, but is undoubtedly greatly retarded in comparison with the mutation occurring in plain broth. It is interesting to note that in the beef infusion-serum mixture, only one Type G

TABLE III.
D → G Mutation in Beef Infusion and Other Media.

Period of incubation at 37°C.	Proportion of Types D and G.					
	Tube No.					
	1	2	3	4	5	6
	6-hr. Type D filtrate.	Plain broth.	5 per cent serum broth.	Beef infusion.	5 per cent serum-beef infusion.	Undiluted serum.
<i>hrs.</i>						
12	D	D	D	D	D	D
24	"	"	"	"	"	"
36	"	"	"	"	"	"
52	"	D + few G.	"	"	"	"
84	"	" + " "	"	"	"	"
96	D + 3 G.	D + moderate number of G.	D + 1 G.	"	"	"
120	" + few G.	D + many G.	D	"	"	"
144	" + few G.	D + G (70:30).	"	D + 1 G.	"	"
173	" + few G.	D + G (30:70).	D + many G.	D	"	"
197	D	D + G (30:70).	D + many G.	"	D + 1 G.	"
228	"	D + G (40:60).	D	"	D	"
<i>days</i>						
18	"	D + moderate number of G.	"	D + G (95:5).	"	D + few G.

colony was observed over a period of 18 days. The 6 hour Type D filtrate showed its usual inhibitory activity. This was the same filtrate as that used in Experiment 2. It had been kept in the ice box for 9 days after that experiment. In undiluted rabbit serum no Type G colonies were observed for 228 hours. A few were revealed by the examination after 18 days. This indicates, again, that the process

D \rightarrow G takes place in a variety of media, but that the tendency is far more marked in some than in others.

A further interesting fact is to be observed in Table III. It will be noted that in Tube 2 (plain broth) the proportional number of Type G colonies reaches a maximum after 173 to 197 hours, is less in 228 hours, and far less after 18 days. This is also true of the serum broth (Tube 3). This phenomenon has been observed many times and its analysis is demanded. The great decrease in the relative number of Type G colonies must be due to one of two causes. Either the mutant G forms die off more rapidly than do the parent D microbes, or an equilibrium is established in the tubes which results in a tendency of the newly mutated Type G to revert to the original Type D. Such reversion has never been noted in subcultures made by fishing Type G colonies. On the other hand, it is conceivable that an equilibrium might be set up in the original reaction tube.

TABLE IV.

D \rightarrow G Mutation in 2 Per Cent Peptone (Fairchild's).

Period of incubation at 37°C., hrs.	15	30	48	72	96	134	146	183
Proportion of Types D and G.	D	D + 3 G.	D	D	D + G (40:60).	D + few G.	D	D

The striking retardation of D \rightarrow G mutation in beef infusion led to the examination of peptone as a possible factor in the hastening of the process. Preliminary experiments indicated that it was necessary in order to obtain growth to seed relatively large amounts of Microbe D into the peptone solutions.

Experiment 4. D \rightarrow G Mutation in 2 Per Cent Peptone (Fairchild's).—The peptone solution was made up in distilled water and titrated to reaction pH 7.4. A large test-tube containing 10 cc. of this medium was then seeded with 0.5 cc. of a 10 hour culture of pure-line strain, Type D, in peptone. The tube was incubated at 37°C. for 183 hours and tests on serum agar plates were made at intervals during this time. The result is given in Table IV.

Marked mutation is seen to occur in peptone solution, and the phenomenon of a decrease in the number of Type G colonies after a maximum is strikingly manifest in this experiment.

TABLE V.
D → *G* Mutation in Various Concentrations of Peptone (Fairchild's).

Tube No.	Medium.			Proportion of Types D and G.						
	Peptone 20 per cent.	Beef in-fusion.	Final per cent of peptone.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.	192 hrs.
	cc.	cc.								
1	10.0		20.0	D	D + 7 G.	D + 10 G.	D + G (50:50).	G + few D.	G + 2 D.	D + G (50:50).
2	5.0	5.0	10.0	D + 3 G.	D	D + many G.	D + G (90:10).	D + G (10:90).	D + G (10:90).	D + G (50:50).
3	2.5	7.5	5.0	D	D + G (90:10).	D + G (40:60).	D + G (15:85).	D + G (2:98).	D + few G. (2:98).	D + G (50:50).
4	0.5	9.5	1.0	"	D	D	D	D + few G.	D + few G.	D
5	0.1	9.9	0.2	"	D + 3 G.	"	"	" + " "	D	"
6		10.0		"	D	"	"	" + 2 G.	"	"
7	Plain broth.			"	"	D + 8 G.	"	D + many G.	D + many G.	D + few G.
8	Rabbit serum undiluted.			"	D + few G.	D	D + 1 G.	D	D	D

The next experiment dealt with the effect of various concentrations of peptone on the process. A stock solution of 20 per cent Fairchild's peptone was prepared and titrated to pH 7.4.

Experiment 5. D \rightarrow G Mutation in Various Concentrations of Peptone (Fairchild's).—Eight large tubes were prepared containing respectively 20 per cent peptone solution undiluted, 20 per cent peptone solution diluted to varying degrees with beef infusion, beef infusion alone, plain broth, and undiluted rabbit serum. The pH of all of these media was 7.4. They were seeded with 0.1 cc. of a pure-line strain of Microbe D, 6 hour culture in rabbit serum, which showed only Microbe D when streaked on serum agar. The tubes were then placed in the incubator at 37°C. and streaked on serum agar plates at intervals up to 8 days. The results of the experiment are given in Table V.

Table V shows in a very striking manner the marked effect of high concentration of peptone on the mutation. The process occurs much more rapidly than is usually the case in plain broth, and, what is more, in the case of 20, 10, and 5 per cent peptone, goes much nearer to completion. That is to say, the mutation instead of involving 30 to 40 per cent of the organisms, reaches 90 per cent and in some cases arrives almost at completion. On the other hand, the tubes with very low concentrations of peptone or with beef infusion alone and with undiluted rabbit serum show a very few Type G colonies during the entire period of the experiment and in the last two tests proved to contain nothing but Type D. The decrease in relative number of Type G colonies is again strikingly apparent in Tubes 2 and 3, 192 hour test.

It cannot be said that the presence of the peptone causes the mutation D \rightarrow G, since the change occurs occasionally, though only to a small extent, in beef infusion and in undiluted rabbit serum. But it is certain that the presence of peptone in suitable concentrations greatly accelerates a reaction toward which a tendency already exists. It is interesting that four pure-line strains, kept on ice in undiluted rabbit serum for 3 months without passage, showed no evidence of the appearance of Type G colonies when subsequently transplanted.

Some preliminary experiments have been made in regard to the effect of varying C_{H+} of the medium upon the D \rightarrow G process. These of course are limited by the range within which good growth occurs; *i.e.*, about pH 8.5 to pH 6.0. The results would indicate that

reaction of pH 6.0 distinctly retards the process, while it is, if anything, accelerated in reaction pH 8.5.

DISCUSSION.

Great confusion exists among bacteriologists in regard to the meaning of the term mutation. Its use in application to the experiments just described is considered to be entirely justified in the light of the definition of Dobell (4), who speaks of it as "a permanent change—however small it may be—which takes place in a bacterium and is then transmitted to subsequent generations." The Type G colonies arising in these experiments, and subcultured to undiluted rabbit serum, were frequently tested for the characters that distinguish them from the parent Type D. The mutant G forms were found in every case to possess little or no virulence, to grow in a granular fashion in fluid media, and to possess an acid agglutination point distinctly different from that of Type D. All of these characters persist throughout many passages in undiluted serum, a medium in which the original change seldom, if ever, takes place. It would seem that if a so called atavistic reversion were to take place, it would certainly occur in undiluted serum. This has never been observed.

The majority of authentic examples of bacterial mutation has been concerned with gain or loss in fermentative power. We refer here only to work performed with pure-line strains. Among these researches, the investigations of Benecke (5) and Kowalenko (6) deserve to be mentioned. These investigators found that Massini's (7) *Bacterium coli mutabile* gained the power of splitting lactose when cultivated in this sugar and that this power persisted indefinitely. The work of Müller (8) in regard to *Bacillus typhosus* is of a similar nature as is that of Burri (9) with *Bacterium imperfectum*. It would seem that the D \rightarrow G process just described involves a much more fundamental change in the bacterial cell. The distinct difference in acid agglutination optimum is a remarkably regular occurrence. It is in the nature of a physical constant for each type and would imply an important change in the protoplasm of the organism. It is certain that this phenomenon is not confined to the hemorrhagic septicemia group, of which the rabbit septicemia bacillus is a member. Varieties showing granular growth character have been discovered in

typical cultures of paratyphoid organisms by Krumwiede (10), of *Bacillus dysenteriae* Shiga by Arkwright (11) and Zoeller (12), and in cultures of *Bacillus coli* by Gratia (13). The way in which these have arisen has not been taken up by these authors, but it is quite certain to be found of a nature closely akin to the process just described.

SUMMARY AND CONCLUSIONS.

Type G microbes, discovered in pure cultures of the rabbit septicemia bacillus, have been demonstrated to arise from the parent D form by mutation.

The $D \rightarrow G$ mutation takes place in broth cultures of pure-line strains of Microbe D, when these are kept for several days without transplant at 37°C., or at room temperature, or in the ice box.

The mutation is greatly inhibited by filtrates from 6 and 24 hour cultures of Microbe D, and to some extent by filtrates from 48 hour cultures.

The process of transformation takes place to a very slight extent or not at all in undiluted rabbit serum, but Type G colonies subcultured to this medium do not revert to the parent D form.

The $D \rightarrow G$ change is strongly inhibited in cultures made in simple beef infusion, or in 5 per cent rabbit serum-beef infusion.

Peptone would seem to be the constituent of plain broth which favors the process. In high concentrations of peptone, the mutation is rapid and may reach a degree of 90 per cent of the total organisms in 5 to 6 days.

A distinct maximum of the relative number of Type G colonies as compared to the parent Type D is observable in plain broth and in some concentrations of peptone, when these are kept at 37°C. for some days without transplant. Subsequent tests show the concentration of Type G microbes to diminish.

The change in acid agglutination optimum exhibited by the mutant G forms implies a distinct change in bacterial protoplasm and would seem to be one of the most fundamental mutations so far described.

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EXPLANATION OF PLATE 38.

FIG. 1. 10 per cent rabbit serum agar plate showing mixed culture of Microbes D and G. Type G preponderates and has a grayish appearance. The Type D colonies are white. The arrows indicate type D colonies.

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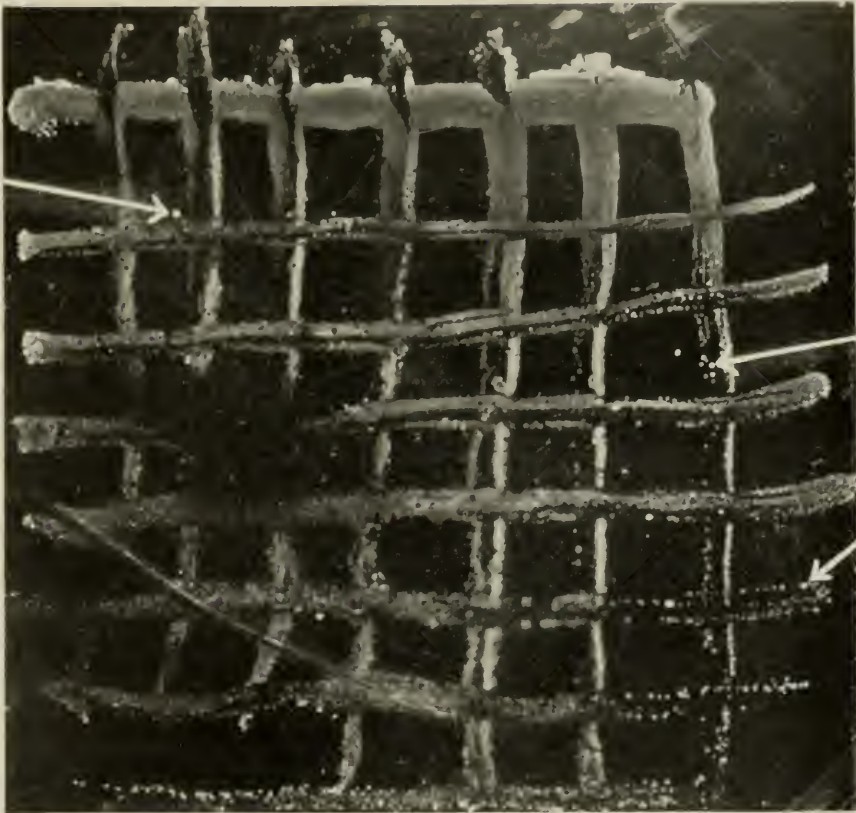


FIG. 1.

(De Kruif: Mutation of bacillus of rabbit septicemia.)

SPONTANEOUS DECREASE OF THE SURFACE TENSION OF SERUM. I.

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When a serum is exposed to the air, the surface energy of its free surface decreases progressively, according to a certain law, until after about 10 minutes a state of equilibrium is attained. The purpose of this paper is to report a study of this phenomenon, which has been overlooked so far on account of lack of proper apparatus for measuring surface tension.

The existence of a film at the surface of protein solutions has been discussed by Ramsden.¹ He pointed out the formation of semisolid or highly viscous films, and merely noted that "the matter which accumulates [in the surface layer] possesses the property of lowering the surface tension . . . of the free surface of water."¹ The technique which he used did not allow him to proceed further in that line, and he neither observed nor measured any lowering in the surface tension of the serum itself, not did he notice the variations of the surface tension of water plus serum in function of the time. There is no doubt but that the existence of a more concentrated layer at the free surface or at all interfaces plays an important part in most biological phenomena. Whether this "film" is solid or liquid with simply higher viscosity will be discussed later. In order to prevent any misunderstanding, the word film will only be used to define a liquid film; the term "membrane" will be used with reference to a solid film.

I.

EXPERIMENTAL.

Over 3,000 measurements of surface tension were made. The instrument used was the tensiometer, previously described.² The

¹ Ramsden, W., *Proc. Roy. Soc. London*, 1904, lxxii, 156.

² du Noüy, P. L., *J. Gen. Physiol.*, 1918-19, i, 521.

use of this apparatus is based upon the adhesion of a platinum ring to the surface of the liquid, and the stress necessary to tear off the film is measured by the torsion of a steel wire. A measurement with this instrument requires approximately only 20 seconds, and the instrument always measures the tension of the same layer of liquid.

The ring method was adopted, after much experimenting, as the only reliable one for colloidal solutions, as well as the simplest, quickest, and most accurate. This opinion is now shared by a large number of physicists, and particularly by Ferguson, who reported about twenty different techniques for measuring surface tension.³

The tensiometer⁴ was used with an accurately calibrated platinum-iridium ring (circumference 4.00 cm.), and is reliable to ± 0.1 dyne. We believe that any data giving the second and third decimals are useless in general because, so far, none of the twenty odd methods in existence check each other, some differing by more than 5 dynes for the same substance at the same temperature, and because no theoretical reasons exist yet for adopting a standard value of surface tension for any substance.

All measurements were made between 23° and 25°C., room and liquid temperature. All glasses, containers, and pipettes were boiled in cleaning solution (sulfuric acid and sodium dichromate), then rinsed in distilled water, and dried. The free surface of the liquid was always 10 sq. cm. with a tolerance of ± 0.5 sq. cm. The elapsed time between the pouring of the liquid and the measurement was very nearly constant, and equalled 15 seconds. The ring was washed in distilled water and flamed after every measurement. The same precautions were taken in collecting and preparing the serum. Only clean glass was used. No alcohol or ether was ever employed. When it occasionally happened that the edge of a tube had been touched with the fingers, a marked difference was observed in the readings.

The results are shown in Table I.

Text-figs. 1 and 2 show more clearly the progressive decrease of the surface tension. It is quite remarkable that in 2 minutes the drop can be of such amplitude as 2 dynes. In the three cases shown in

³ Ferguson, A., *Science Progr. 20th Cent.*, 1914-15, ix, 428.

⁴ Made by the Central Scientific Co. of Chicago.

Table I, the drop was very nearly 2.1 dynes. In 20 minutes, the surface tension was decreased by 4.1 and 3.3 dynes for the old dogs, and by 5.6 dynes for the very young dog. All three reached their equilibrium at about the same value.

If the serum is stirred at the end of the experiment, that is, when the surface tension seems to have attained a stable value, the homogeneity of the serum is momentarily reestablished, the surface tension rises

TABLE I.
Serum 1020 (Dog 12 Yrs. Old) (Text-Fig. 1).
Temperature 25°C.

Time.	0	2 m.*	6 m.	12 m.	20 m.	30 m.
Surface tension, dynes.....	59.6	57.5	56.9	56.1	55.5	55.5

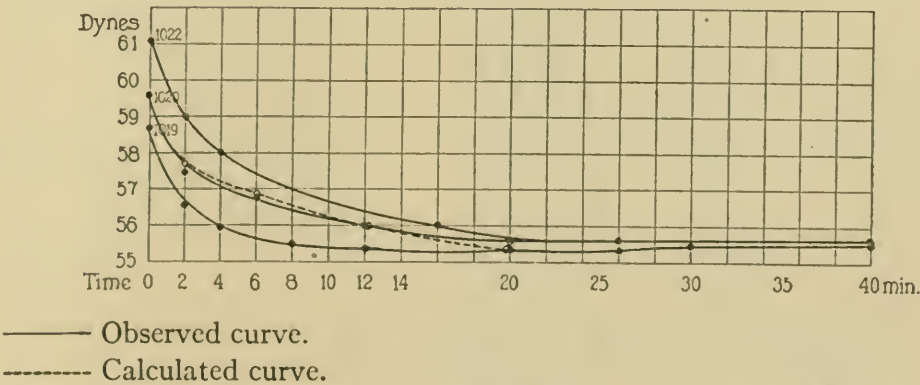
Serum 1022 (Dog 1 Yr. Old) (Text-Fig. 1).
Temperature 23°C.

Time.	0	2 m.	6 m.	16 m.	25 m.	40 m.
Surface tension, dynes.....	61.1	59.0	57.8	56.0	55.6	55.6

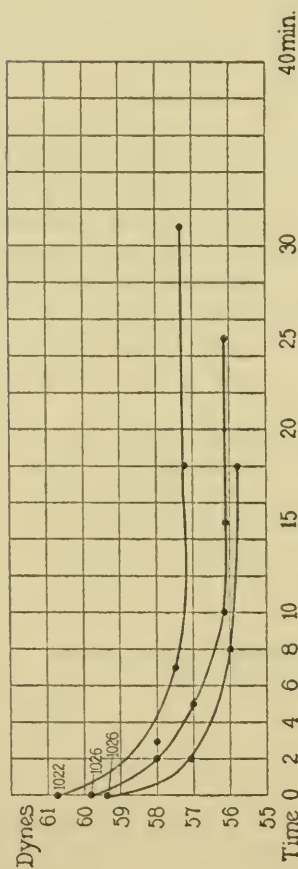
Serum 1019 (Dog 12 Yrs. Old) (Text-Fig. 1).
Temperature 23°C.

Time.	0	2 m.	4 m.	8 m.	10 m.	20 m.	25 m.	30 m.	40 m.
Surface tension, dynes.	58.7	56.5	56.0	55.5	55.4	55.4	55.5	55.4	55.6

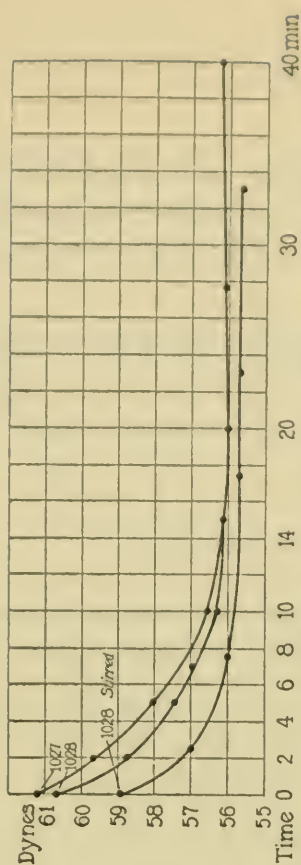
* In Tables I to X, the abbreviations h., m., and s. are used for hours, minutes, and seconds.



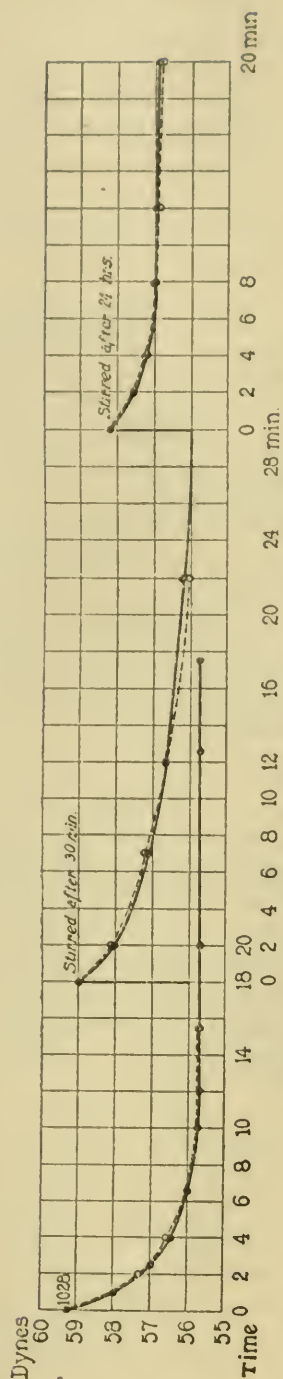
TEXT-FIG. 1. Modification of the surface tension of serum in function of the time.



TEXT-FIG. 2. Modification of the surface tension of serum in function of the time. Two samples of the same serum (No. 1026) were kept 4 days in test-tubes, the first in the ice box (initial value 59.4 dynes), the second at room temperature (22°C.; initial value 59.8 dynes).



TEXT-FIG. 3. Modification of the surface tension of serum in function of the time.



TEXT-FIG. 4. Effect of stirring on the surface tension of serum.

immediately, and the liquid recovers its property of forming another film. However, something is changed; the surface tension immediately after stirring does not reach its original value, and generally does not drop as much or as rapidly (Table II and Text-fig. 3). Text-fig. 4 shows plainly the change in the amplitude and in the rate of the phenomenon; the slope of the third curve (after 24 hours) is decidedly less marked; $\frac{d\gamma}{dt}$ decreases progressively.

TABLE II.
Serum 1027 (Dog 8 Yrs. Old).
Temperature 24°C.

Time.	0	2 m.	4 m.	8 m.	13 m.	23 m.	33 m.	4 h
Surface tension, dynes.....	59.8	58.5	57.9	57.1	56.5	56.5	56.3	56.3

Same serum stirred after 4 hrs., 59.0 dynes.

Serum 1028 (Dog 10 Yrs. Old) (Text-Figs. 3 and 4).
Temperature 24°C.

Time.	0	2 m.	7 m.	17 m.	27 m.	37 m.
Surface tension, dynes.....	59.3	57.1	56.0	55.7	55.7	55.7

Same serum stirred with a glass rod a few times, after 30 min., 59.0 dynes.

Time.	0	2 m.	4 m.	9 m.	14 m.	24 m.	50 m.	2 h.	4 h.	24 h.
Surface tension, dynes...	59.0	58.0	58.0	57.1	56.9	56.0	56.0	55.4	56.0	55.4

Same serum stirred again, after 24 hrs., 58.2 dynes.

Time.	0	2 m.	4 m.	8 m.	20 m.	30 m.
Surface tension, dynes.....	58.2	57.6	57.3	57.0	56.9	56.9

The results were comparable in experiments on chicken serum (Table III).

An interesting fact, which may be considered as a good control, is that these two sera taken from similar chickens and showing at the beginning a difference of only 0.2 dyne in their surface tension have kept this difference practically constant during 50 hours, and after

TABLE III.

*Serum 1053 (Chicken).**Temperature 25°C. Oct. 11, 1921.*

Time.	0	2 m.	4 m.	6 m.	11 m.	16 m.	26 m.	41 m.	56 m.
Surface tension, <i>dynes</i> .	62.0	61.2	61.0	61.0	60.7	60.0	58.0	57.0	57.0

Temperature 25°C. Oct. 12, 1921.

Time.	20 h.	Stirred.	24 h.
Surface tension, <i>dynes</i>	54.0	57.5	53.0

Temperature 25°C. Oct. 13, 1921.

Time.	44 h.	Stirred.	47 h.	50 h.
Surface tension, <i>dynes</i>	53.2	53.2	53.6	53.6

*Serum 1054 (Chicken).**Temperature 25°C.*

Time.	0	3 m.	6 m.	11 m.	20 m.	35 m.	1 h. 35 m.	16 h.
Surface tension, <i>dynes</i>	62.1	61.5	61.2	60.2	60.0	59.5	58.0	54.2

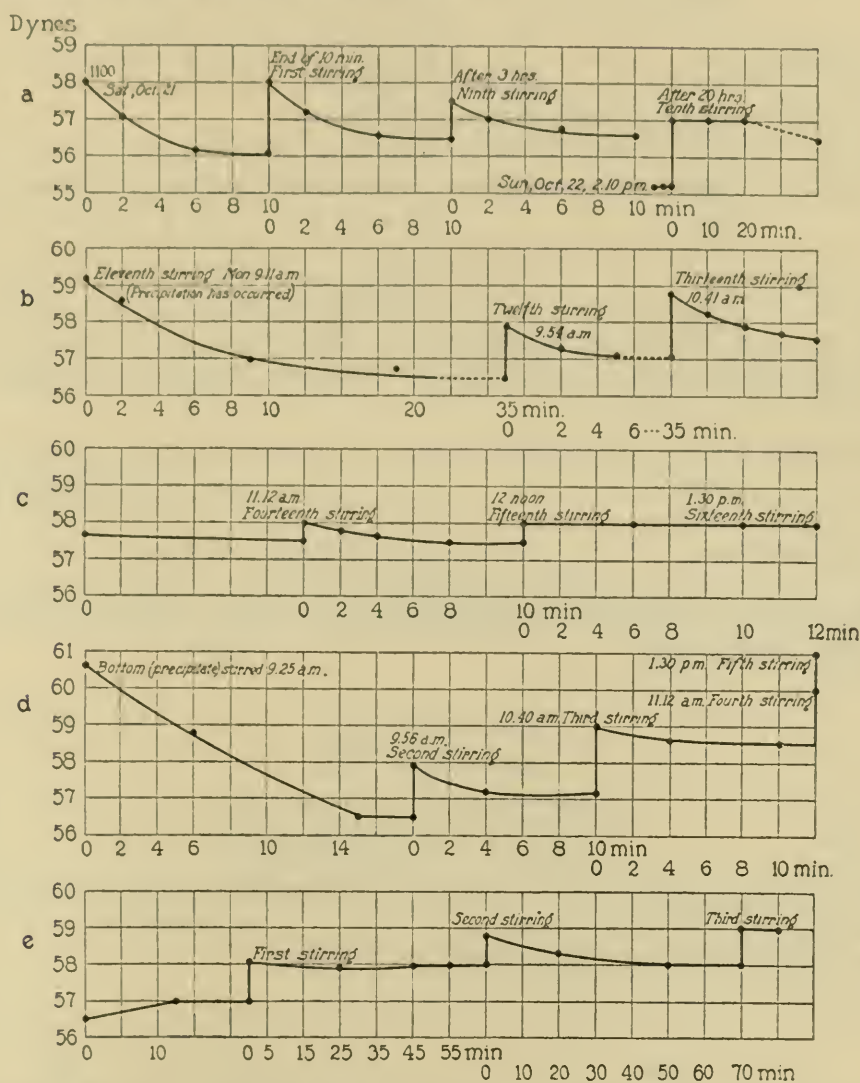
Serum stirred after 16 hrs., 58.0 dynes.

Time.	0	2 m.	12 m.	22 m.	38 m.	1 h. 10 m.	1 h. 40 m.	3 h. 55 m.	6 h.	7 h.
Surface tension, <i>dynes</i> ..	58.0	58.0	57.1	56.9	56.0	55.0	54.6	53.2	54.6	54.6

Total time elapsed, 24 hrs. On Oct. 13, 1921, after 40 hrs., 53.0 dynes.
Stirred, 57.0 dynes.

Time.	0	3 h.	5 h. 30 m.
Surface tension, <i>dynes</i>	57.0	54.0	53.9

being reactivated by stirring more than once. At the end of about 20 hours, the first was 54.0, and the second 54.2; at the end of 24 hours (after stirring), 53.0 and 53.2; at the end of 50 hours, 53.6 and 53.9, the difference here having become 0.3.



TEXT-FIG. 5, *a* to *e*. (*a* to *d*) Effect of stirring on the surface tension of serum. (*e*) Behavior of a control sample of the same serum, left unstirred during the 2 days, and stirred on Oct. 23, at 4 p.m., for the first time (precipitate at the bottom). Original value on Oct. 21, 56.5 dynes.

The rise in surface tension which follows the redissolution of the liquid surface layer by stirring is a general phenomenon with all clear, fresh sera. The progressive decrease in the activity, which can be compared graphically to a damping effect, seems constant also (Text-fig. 5, *a* to *e*). But it often happens that after 24 hours the property

TABLE IV.
Serum 111 (Rabbit).
Temperature 23°C.

Time.	0	2 m.	15 m.	25 m.	55 m.	1 h. 35 m.
Surface tension, dynes.....	57.5	56.5	55.5	55.4	54.7	54.6

Serum stirred after 1 hr. and 35 min., 56.9 dynes.

Time.	0	2 m.	6 m.	11 m.	26 m.	44 m.
Surface tension, dynes.....	56.9	56.1	55.5	54.6	55.0	54.7

After 2 hrs. and 20 min., the surface tension was 54.7 dynes. The next morning, after 18 hrs. and 20 min., it was 53.6 dynes. Stirred, it rose to 55.1 dynes.

Time.	0	2 m.	10 m.	1 h. 15 m.	2 h. 15 m.	2 h. 20 m.
Surface tension, dynes.....	55.1	55.0	55.0	55.1	55.0	55.0

Serum stirred at beginning of experiment, 55.1 dynes. Stirred after 2 hrs. and 20 min., 55.0 dynes.

Serum 1060 (Dog).
Temperature 23°C.

Time.	0	2 m.	6 m.	13 m.	23 m.	38 m.	2 h. 8 m.	3 h.	3 h. 15 m.
Surface tension, dynes.	59.3	57.5	56.8	56.0	55.5	55.4	56.0	56.0	56.0

Serum stirred after 3 hrs. and 15 min., 58.0 dynes.

Time.	0	2 m.	5 m.	10 m.	40 m.
Surface tension, dynes.....	58.0	57.1	57.0	56.7	56.0

Time.	0	2 m.	6 m.	14 m.	19 m.	50 m.	1 h. 20 m.
Surface tension, dynes.....	56.2	55.6	55.6	55.6	55.6	55.6	56.0

After 18 hrs. the surface tension was 54.2 dynes; stirred, 56.0 dynes; stirred again after 19 hrs. and 20 min., 56.0 dynes.

is completely lost. The serum is inactivated from this standpoint and its surface tension remains constant (Table IV). As will be observed, the decrease still continued after the 4th hour, and over night it dropped from 56 to 54.2 dynes. At the end of the experiment, the serum became too viscous and had to be discarded.

Action of Heat.

Time is not the only factor which inhibits this activity of the serum. Heat acts in the same way; it may not act definitively by destroying

TABLE V.
Serum 1020 (Dog; Clear, Viscous).
Temperature 25°C. Heated for 20 Hrs. at 55°C.

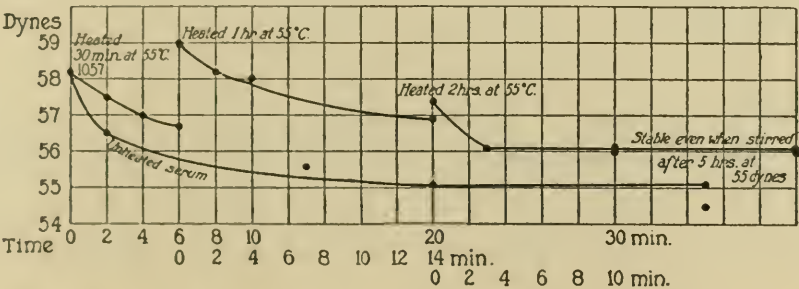
Time.	0	2 m.	8 m.	18 m.	33 m.
Surface tension, dynes.....	56.0	56.0	56.0	55.7	55.8

Serum 1021 (Dog; Cloudy).
Temperature 24°C. Heated for 20 Hrs. at 55°C.

Time.	0	2 m.	5 m.	11 m.	20 m.
Surface tension, dynes.....	57.5	57.1	57.3	57.3	57.3

Serum 1022, Control (Dog; Clear).
Temperature 24°C. Unheated.

Time.	0	3 m.	6 m.	11 m.	17 m.	31 m.
Surface tension, dynes.....	60.8	58.0	57.5	57.4	57.1	57.3



TEXT-FIG. 6. Action of heat on the surface tension of serum.

the substance, but the behavior of the serum is modified profoundly at the start, as will be seen in Table V.

TABLE VI.
Serum 1057 (Dog; Clear) (Text-Fig. 6).
Temperature 24°C. Heated for 30 Min. at 55°C.

Time.	0	2 m.	4 m.	8 m.
Surface tension, dynes.....	58.2	57.5	57.0	56.7

Temperature 24°C. Heated for 1 Hr. at 55°C.

Time.	0	2 m.	4 m.	14 m.
Surface tension, dynes.....	59.0	58.2	58.0	56.9

Temperature 24°C. Heated for 2 Hrs. at 55°C.

Time.	0	3 m.	5 m.	10 m.	25 m.	55 m.
Surface tension, dynes.....	57.4	56.1	56.1	56.1	56.1	56.1

Serum stirred after 55 min.; surface tension 61.0 dynes.

The Same Sample, after 20 Hrs. Temperature 24°C.

Time.	0	2 m.	9 m.	19 m.	34 m.
Surface tension, dynes.....	61.0	60.7	60.0	59.5	59.2

Temperature 24°C. Heated for 3 Hrs. at 55°C.

Time.	0	10 m.	45 m.	1 h. 15 m.	2 h. 15 m.	3 h.
Surface tension, dynes.....	59.3	57.0	57.0	57.4	57.5	57.4

Apparently, at least 5 hours heating at 55°C. were necessary to inactivate the sera completely, although after 2 hours the activity was reduced, as shown by Table VI and Text-fig. 6. In this case, the control itself was inactivated after 24 hours without heating.

Effect of Precipitation of Insoluble Substances in the Serum.

All the sera in which a precipitation of insoluble substances had occurred, in the form of a coarse colloidal suspension showing a tendency to settle out spontaneously after a few hours standing, were devoid of any activity (Table VII). But if the precipitate was allowed to settle, after the liquid had been stirred, the surface tension was decreased.

TABLE VII.

*Serum 1059 (Dog; Cloudy; Precipitate in Suspension).
Temperature 23°C.*

Time.	0	2 m.	5 m.	32 m.
Surface tension, dynes.....	53.8	53.8	53.6	53.6

When a serum is allowed to dry entirely and is redissolved in plain water, it still manifests the same activity, although to a smaller extent (Table VIII and Text-fig. 7).

TABLE VIII.

*Serum 1027 (Dog). Dried and Dissolved in Water (Text-Fig. 7).
Temperature 24°C.*

Time.	0	2 m.	5 m.	12 m.	27 m.	42 m.	1 h. 27 m.	2 h. 50 m.
Surface tension, dynes.....	58.0	57.2	57.2	56.9	56.1	56.2	56.2	56.1

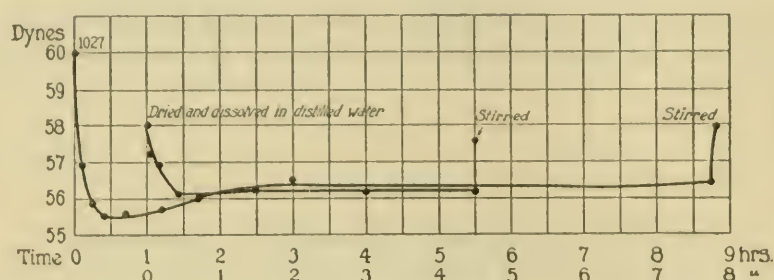
*Serum 1027, Control.
Kept at Room Temperature for 48 Hrs. (23°C.).*

Time.	0	2 m.	4 m.	7 m.	12 m.	22 m.	45 m.	1 h. 15 m.	1 h. 30 m.	2 h.	2 h. 15 m.	2 h. 30 m.	2 h. 45 m.	3 h. 45 m.	8 h.
Surface tension, dynes..	60.0	57.5	57.0	56.1	55.9	55.5	55.6	55.7	56.0	56.1	56.2	56.5	56.2	56.5	56.5

Same serum stirred, 58.0 dynes.

Great care is necessary when taking the measurements, for the slightest agitation of the surface causes the film to dissolve, with a

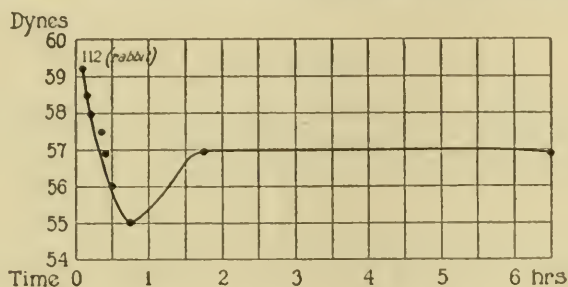
consequent rise in the surface tension. The first series of measurements was more carelessly done, and gave results which did not check with the later ones. We had to make a set of very careful measurements in which the liquid was absolutely undisturbed, to



TEXT-FIG. 7. Surface tension of fresh serum, and of the same sample dried and dissolved in water.

find out the reason for such unaccountable facts as a spontaneous rise after a drop, such as that shown in Text-fig. 8.

Text-fig. 5, *e* illustrates the damping effect again, and also shows that the progressive inactivation is mainly function of the time and



TEXT-FIG. 8. Spontaneous rise in surface tension of serum after a fall. The rise after 40 minutes and after $6\frac{1}{2}$ hours was due to the careless handling of the watch-glass containing the serum.

is but little affected by stirring. It illustrates also the progressive increase—reverse phenomenon—which takes place after each stirring, when precipitation has occurred in the serum. This is probably due, as pointed out elsewhere, to the formation of a membrane or semisolid film in the surface layer (see also Text-fig. 5, *d*).

II.

DISCUSSION.

The hypothesis of surface coagulation, or the formation of an homogeneous solid film, does not seem to us to account for any of the phenomena studied. First, there is no sign, optically, of such a film. Moreover, a solid film would increase, rather than decrease, the surface tension; and finally, it would not account for the damping of the phenomenon, in function of the time. Besides, by very light stirring the tension rises again, and most of the sera experimented upon remained perfectly clear, although their viscosity increased, even after 48 hours.

An objection may be made that temperature and evaporation (concentration) play a part in this phenomenon. But a decrease in temperature, which actually occurs as soon as a liquid is spread over a larger surface, would mean an increase in surface tension, whereas we observe the opposite. Concentration following evaporation requires a good deal of time; after 20 minutes, samples were tested by measurements of their cryoscopic point and did not show any marked concentration. As the phenomenon usually takes place in less than 15 minutes, it may be concluded that it can only be affected by the change in the concentration of the surface layer. With highly concentrated sera, evaporated *in vacuo* and reduced to one-third of their original volume, the figures were practically the same.

A few hypotheses are hence possible. Either the drop in surface tension is due to a chemical change and adsorption of the resulting substances in the surface layer, or to an adsorption of bodies generated or contained in the bulk, or else to modifications in the arrangement of the group molecules in the surface layer, or possibly to a combination of these causes. Without making any attempt to discuss the structure of the surface layer, the facts may be accounted for in the case of the first hypothesis by assuming that the huge, fragile protein molecules begin to disintegrate and yield substances acting on surface tension as soon as they are no longer entirely surrounded by others in the liquid, on account of the change in potential energy. In the process of breaking down, they may also liberate progressively

other molecules, the accumulation of which would inhibit the phenomenon by concentrating in the surface layer and decreasing definitively the surface tension.

But it is not necessary to assume that the substances which lower the surface tension are produced in the surface layer. Indeed, the reaction occurs as if these substances came slowly from the bulk of the liquid. An exceedingly slight change in the concentration of certain substances in the serum could explain the facts. But there must be some chemical change to account for the damping effect, and for the inactivation, unless it is attributed to adsorption by colloidal particles. This being plainly a case of adsorption, let us consider what the differences are between this phenomenon and the ordinary phenomenon of adsorption.

In the ordinary case of adsorption, when a substance lowering the surface tension is added to the liquid the value for the surface tension corresponds to the concentration. Equilibrium is usually rapidly established with respect to the quantity adsorbed in the surface layer in excess of the quantity in the bulk, and the surface tension may reach a very low value.

In the present case, on the contrary, all constituents are coexistent, so that, at first sight, the concentration seems constant, as the substance lowering the surface tension exists normally in the serum. But either a reaction or a migration takes place in function of the time, which progressively changes the ratio of concentrations between the bulk and the surface layer. From 10 to 20 minutes are required to reach the equilibrium, which takes place generally between the limits 53 and 56 dynes, never below 53.

In the ordinary case of adsorption, the time taken for attainment of equilibrium is shortened by shaking. In the case of serum, conversely, shaking stops the process, destroys the film, and brings back the original surface tension, almost to its former value. The time required for attainment of equilibrium is not shortened, but lengthened.

Why substances lowering surface tension remain adsorbed in the surface layer, instead of remaining in the bulk as do the bodies which raise surface tension, is theoretically accounted for by the dynamical

theory of solutions, as developed mainly by Gibbs⁵ and Thomson.⁶ It may be of interest to the reader to recall this theory briefly.

The most stable arrangement of any solution must be one accompanied by minimal surface tension. This follows from the simple study of Gibbs' equation:

$$(1) \quad c \frac{d\gamma}{dc} = -u \frac{dp}{dc}$$

in which u is the quantity adsorbed, p the osmotic pressure, c the concentration, and γ the surface tension. For dilute solutions, the osmotic pressure is given by the equation

$$(2) \quad p = RT$$

in which R is the constant of gases and T the absolute temperature. Therefore,

$$(3) \quad dp = RTdc$$

By introducing this value in (1),

$$(4) \quad u = -\frac{c}{RT} \cdot \frac{d\gamma}{dc}$$

As stated above, u is precisely the adsorption; that is, the difference between the concentrations of the surface layer and the bulk. It is called positive when the concentration in the surface layer is greater, and negative when it is smaller than in the bulk. $\frac{d\gamma}{dc}$ is the differen-

tial coefficient of the function connecting surface tension and concentration, and is positive if γ and c increase together and negative if γ decreases with increasing c . Therefore, the whole product on the right hand of the equation will be *negative* in the first case (when the solute *increases* surface tension) and *positive* in the second (when the solute *reduces* surface tension). This means a lower concentration in the surface layer (smaller amount adsorbed, u) in the former case, and a higher concentration in the surface layer in the latter; R , T ,

⁵ Gibbs, W., *Tr. Conn. Acad.*, 1878, iii, 380; Scientific papers, London, 1906.

⁶ Thomson, J. J., *Applications of dynamics to physics and chemistry*, London, 1888, 191.

and c being necessarily positive, the sign of u depends only on that of $\frac{d\gamma}{dc}$.

This explains why Gibbs' law is often given under the following form. A small amount of dissolved substance may reduce the surface tension considerably, but can only increase it slightly.

It is possible to account not only for the decrease in surface tension represented by the first curves, but also for the damping of the phenomenon, by assuming that some kind of chemical or physicochemical change is undergone by the substances in solution in the serum, in function of the time. The action of these substances, which would accumulate slowly, would be superimposed on that of the bodies acting normally, and would progressively lower the initial value of the surface tension. The presence of these new substances, whatever they are, would forcibly change the physicochemical qualities of the serum. Hence the curve representing this second stage of the phenomenon is slightly modified. The rate of the lowering of surface tension is slower, owing probably to the increased viscosity of the serum with time. When ten stirrings are made within 100 minutes, followed by a lowering of surface tension, the change in the curves, that is, in the rate of decrease, is not very different from that which would have been obtained had the serum been stirred only once at the end of the 100 minutes. Time acts more efficiently than stirring.

When a serum becomes old, precipitation occurs in the surface layer where the concentration is higher, and insoluble substances are made out of soluble ones. These ultramicroscopic particles agglomerate little by little into larger ones, until they are so heavy that they precipitate. They have a tendency to transform the surface layer into a semisolid, semiliquid layer with higher surface tension (Experiment 1059, Table VII). In order to prevent confusion we shall refer hereafter to the reaction which takes place for the first time, as soon as the serum is exposed to the air, as the first reaction. After the first stirring, it will be the second reaction, and so on. There is no doubt that one reaction takes place as soon as the blood is removed from the body, and another every time the serum is poured from one tube to another. But in such cases, the surface exposed is small and when not moved is protected by the first film formed. Besides, we

must arbitrarily choose a starting point, and mathematical study of the phenomenon shows that our assumption is probably correct.

III.

Mathematical Expression of the Phenomenon.

An attempt was made to establish a general equation on a purely empirical basis, which would express the phenomenon in all cases, before and after stirring, with only one coefficient. We found that the exponential formula

$$(5) \quad \gamma = \gamma_0 e^{-Kt^{\frac{1}{2}}}$$

expressed the facts very closely (in the case of dog and rabbit serum), until the phenomenon became asymptotic to a parallel to the axis of time, the ordinate of which is generally around 55.5 dynes. After 10 to 20 minutes, the curve becomes parallel to the axis of time and the phenomenon stops. This fact may be expressed, if required, simply by the addition of a linear term to formula (5). We have, then,

$$(6) \quad \gamma = \gamma_0 e^{-Kt^{\frac{1}{2}}} + At - B$$

t being the time, γ the surface tension at the time t , γ_0 the surface tension at the beginning of the experiment, K , A , and B three constants. In fact, the constants A and B do not modify the law, and the corrective term is only required for the first reaction. After stirring, the first simple formula (5) suffices to account for the facts with close agreement practically to the point when equilibrium is attained. Indeed, the phenomenon is characterized by only one constant, K , generally equal to 0.01, for average old dogs; B is equal to 1.6 (in the example chosen—Serum 1020, Table X), and A to 0.1.

There may be other equations by means of which this law can be expressed, as always occur in cases of logarithmic curves. But the advantage of this exponential form is that it requires only one constant, the meaning of which is clearer, and that it is short and, as is well known, very easy to calculate under the form

$$(7) \quad \text{Log } \gamma = \text{Log } \gamma_0 - K \sqrt{t}$$

Tables IX and X show the agreement between observed and calculated figures. The two curves in Table X were calculated with the simple formula (5). It will be noticed that the constant K decreased rapidly after each stirring, indicating that the serum becomes saturated, or inactivated, after a certain time.

TABLE IX.
Serum 1028 (Dog).
First Reaction.
 $K = 0.01$

Time.	0	2 m.	4 m.	6 m. 5 s.	16 m.	20 m.	40 m.
Surface tension observed, dynes.....	59.3	57.2	56.5	56.0	55.7	55.7	55.7
“ “ calculated, dynes....		57.3	56.6	56.0	55.7	55.7	55.7

These figures are obtained from the 16th min. on, with the complete formula (6). $C = 0.17$ and $A = 1$.

Second Reaction; Stirred after 30 Min. (Text-Fig. 4).
 $K = 0.005$

Time.	0	2 m.	7 m.	12 m.	22 m.
Surface tension observed, dynes.....	59.0	58.0	57.1	56.7	56.1
“ “ calculated, dynes.....		58.1	57.2	56.7	56.0

Third Reaction; Stirred after 24 Hrs. (Text-Fig. 4).
 $K = 0.00325$

Time.	0	2 m.	4 m.	8 m.	12 m.
Surface tension observed, dynes.....	58.2	57.6	57.3	57.0	56.9
“ “ calculated, dynes.....		57.6	57.3	57.0	56.8

The progressive decrease in surface tension after each stirring is shown again in Text-fig. 5, *a*. As stated before, this phenomenon seems to happen only when there is no precipitation in the liquid; as soon as a certain amount of precipitation has taken place, the surface tension, after stirring, rises higher than its former values.

It may seem very premature to try to apply any theoretical formula to this phenomenon, considering the enormous complexity of the

liquid studied, of whose constitution we are so ignorant. However, an interesting fact leads us to believe that it may some day be done successfully. As already noted this phenomenon is one of adsorption in the surface layer. Mere reasoning makes this clear. But a comparison of one of our curves with a curve obtained by Lewis⁷ for the adsorption of sodium glycocholate by a surface of paraffin oil shows a

TABLE X.
Serum 1020 (Dog under 1 Yr. of Age).
 $K = 0.0065$

Time.	0	2 m.	6 m.	12 m.	20 m.	25 m.
Surface tension observed, <i>dynes</i>	59.6	57.5	56.9	56.1	55.5	55.5
“ “ calculated, <i>dynes</i>		57.7	56.9	56.0	55.3	55.3

In this case, the coefficient is much smaller.

Serum 1022 (Old Dog).
 $K = 0.01$

Time.	0	2 m.	6 m.	16 m.	25 m.
Surface tension observed, <i>dynes</i>	61.1	59.0	57.8	56.0	55.6
“ “ calculated, <i>dynes</i>		59.0	57.75	55.8	

remarkable identity despite the entirely different conditions (Text-figs. 9 and 10). A few formulas have been given for expressing the phenomenon of adsorption in function of the quantity adsorbed and of the concentration. It is clear that they could not be used in our case. Among these we may mention the formula quoted by Duclaux⁸ and that of Freundlich,⁹ inaccurate at higher concentrations as shown by Schmidt,¹⁰ who gives a rather complicated exponential formula¹¹

⁷ Lewis, W. C. McC., *Proc. Physic. Soc.*, 1909, xxi, 150; *Phil. Mag.*, 1909, xvii, 466; *Z. Chem. u. Indust. Kolloide*, 1909, v, 91; also cited by Willows, R. S., and Hatschek, E., *Surface tension and surface energy, and their influence on chemical phenomena*, Philadelphia, 1915, 41.

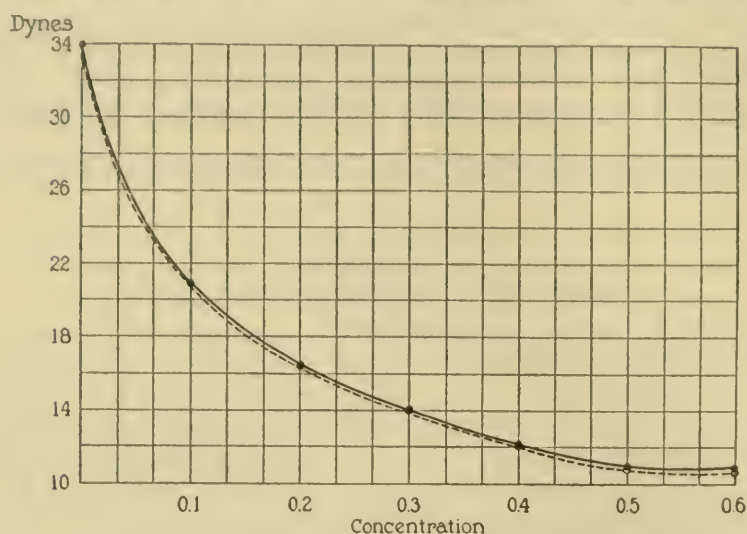
⁸ Duclaux, J., *Les colloïdes*, Paris, 1920, 148.

⁹ Freundlich, H., *Kapillarchemie: Eine Darstellung der Chemie der Kolloide und verwandter Gebiete*, Leipsic, 1909, 146.

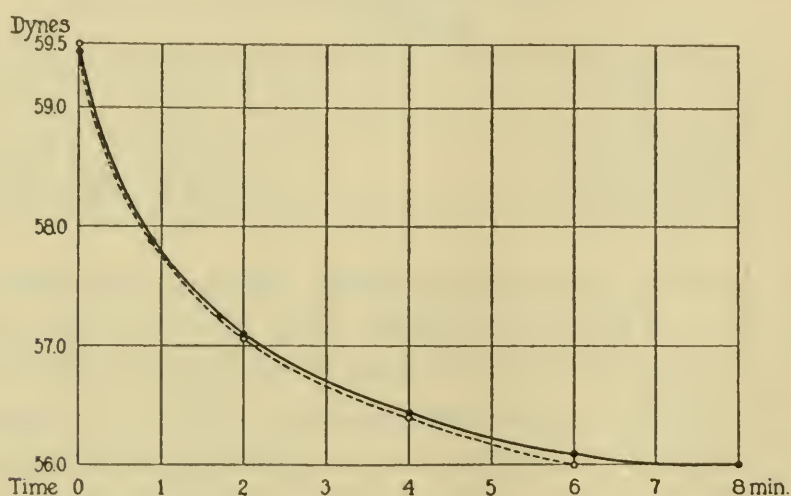
¹⁰ Schmidt, G. C., *Z. physik. Chem.*, 1910, lxxiv, 699.

¹¹ Schmidt, G. C., *Z. physik. Chem.*, 1911, lxxvii, 641.

with two constants. This equation applies well to the adsorption of liquids by solids, and Arrhenius¹² has pointed out that it could be



TEXT-FIG. 9. Action on surface tension of adsorption of sodium glycocholate by paraffin oil (Lewis; adsorption isotherm).



TEXT-FIG. 10. Action on surface tension of adsorption in the surface layer of serum.

simplified. A formula in function of surface tension was given by Sentis¹³ in 1897, and another by Valson in 1872.¹⁴ The last two only

¹² Arrhenius, S. A., *Conférences sur quelques thèmes choisis de la chimie physique*, Paris, 1912, 31.

¹³ Sentis, *Recueil de constantes physiques*, Paris, 1913, 122.

¹⁴ Valson, cited by Morgan, J. L. R., and McKirahan, W. W., *J. Am. Chem. Soc.*, 1913, xxxv, 1759.

apply to inorganic salts, which raise the surface tension and therefore are not adsorbed in the surface layer. Of course, the curve of Lewis is not drawn in function of the time, but in function of the concentration of the solution. In our case, the concentration is constant, but we can admit that the relative concentration is proportional to the time, since the solute which lowers the surface tension is either formed in the free surface layer as soon as it exists, or travels through the bulk to it, in function of the time.

The perfect identity of the curves is emphasized by the fact that the new equation (5), which we proposed for the phenomenon studied in this paper, applies remarkably well to that of Lewis (Table XI). This gives a greater character of generality to this formula, which

TABLE XI.

Agreement between Figures Observed and Calculated for the Lowering of Surface Tension at the Interface between a Solution of Sodium Glycocholate and Paraffin Oil, in Function of the Concentration (Text-Fig. 9).

$$K = 0.222$$

Concentration, <i>per cent.</i>	0	0.1	0.2	0.3	0.4	0.5	0.6
Surface tension observed (Lewis), <i>dynes</i>	34.0	21.0	16.5	14.0	12.2	11.0	10.9
Surface tension calculated ($\gamma = \gamma_0 e^{-Kc^{\frac{1}{2}}}$), <i>dynes</i>		20.2	16.5	14.0	12.2	10.9	9.7

may be applied to the phenomenon studied by Traube,¹⁵ Lewis,⁷ Donnan and Barker,¹⁶ Langmuir,¹⁷ and others, simply by changing t to c ; namely,

$$(8) \quad \gamma = \gamma_0 e^{-Kc^{\frac{1}{2}}}$$

Toward the end of the curve, it becomes parallel to the abscissa of ordinate 10, as a state of equilibrium is attained, and, for the same reasons as expressed above, the calculated data deviate from those observed (Text-fig. 9).

¹⁵ Traube, J., *Kolloidchem. Beihefte*, 1911-12, iii, 237.

¹⁶ Donnan, F. G., and Barker, J. T., *Proc. Roy. Soc. London, Series A*, 1911, lxxxv, 557.

¹⁷ Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

Besides, as the measurements of Lewis were made with the drop weight method, it shows that the method used in our experiments on colloidal substances is as reliable as the most accurate and time-consuming methods used by Lewis.

To give an idea of the order of magnitude of the excess of concentration in the surface layer, since we cannot measure u directly, we may use Gibbs' formula tentatively, without laying emphasis upon the figures given here. Assuming the total concentration in the bulk of a serum to be 9.9 per cent after 1 minute (electrolytes and non-electrolytes), and referring to the formula

$$(4) \quad u = - \frac{c}{RT} \cdot \frac{d\gamma}{dc}$$

we have the values

$$c = 9.9; R = 83.2 \times 10^6; T = 293^\circ; \frac{d\gamma}{dc} = \frac{d\gamma}{dt} = 2$$

$\frac{d\gamma}{dc}$ = the tangent of the angle of the tangent to the point of abscissa 1 with the axis of the abscissæ.

$$u = 0.81 \times 10^{-7} \text{ gm. per sq. cm.}$$

The free surface being about 10 sq. cm., it gives a total amount, roughly, of $\frac{1}{1,000,000}$ gm. of substance adsorbed in the surface layer; but as we deal with colloidal solutions, this figure is almost certainly wrong.

As a point of comparison, it may be said that for sodium glycolate Lewis found experimentally that $u = 3.6 \times 10^{-6}$ instead of about 1.0×10^{-7} , the theoretical value; and Donnan and Barker, using nonylic acid as solute, obtained for u , 0.95×10^{-7} , instead of 0.55×10^{-7} , calculated.

We must not forget that the theoretical values of u (quantity adsorbed) in certain cases were twenty to thirty times smaller than the experimental values. This may be due partly to great difficulties in the technique. The discrepancy is still more marked for colloidal substances. Langmuir¹⁷ has insisted upon the differences existing between theoretical and experimental data.

Further experiments are now being carried out on this subject.

IV.

CONCLUSIONS.

1. Over 3,000 measurements of surface tension of sera have been made with the ring method, and they have yielded a new phenomenon, the spontaneous and rapid decrease of the surface tension of a serum in function of the time.

2. Generally, after 10 minutes the surface tension reaches a value which is practically constant. At least, the decrease is very much slower. After stirring, a rise occurs and a similar phenomenon takes place; but stability is not obtained as rapidly, requiring about 25 minutes. By stirring again, the same thing happens repeatedly, the slope of the curve being less marked each time, the rise in surface tension being slightly below each previous value, and the phenomenon undergoing a sort of damping.

3. An equation was established which expresses the experimental facts with an accuracy of about 0.2 per cent. It applies to the whole phenomenon, before and after stirring. It has only one characteristic constant,

$$\gamma = \gamma_0 e^{-Kt^{\frac{1}{3}}}$$

This formula, by simply changing t to c (concentration), expresses satisfactorily in general the phenomenon of adsorption in the surface layer; that is, the decrease in surface tension in function of the concentration.

4. Prolonged heat, at 55°C., and time seem to inhibit this phenomenon.

5. When precipitation occurs in a serum, the bottom of the liquid, which contains the precipitate, has the highest surface tension. When stirred, the surface tension rises a little every time. The upper part, clear, with lower surface tension, shows the reverse phenomenon; after every stirring, the surface tension becomes a little lower.

A CUTANEOUS NEMATODE INFECTION IN MONKEYS.*

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PLATES 39 TO 52.

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INTRODUCTION.

While attempting to reproduce acute rheumatic fever in monkeys (*Macacus rhesus*), there were observed fever, subcutaneous nodules, and swellings about joints, which closely resembled some of the features of the disease in man. These features were first observed in monkeys inoculated with material from patients with acute rheumatism, but upon closer examination of the entire stock of animals, similar manifestations were occasionally encountered in normal animals and in some that had recovered from measles. In addition to subcutaneous nodules, the most striking lesion, upon casual observation, was a blister on the palms and soles, thought at first to be due to irritation from the disinfectant used in cleaning the cages. The keeper of the animal house stated that he had occasionally noted similar bullæ in monkeys imported in previous years. At first, no connection between these blisters and the subcutaneous nodules and periarticular edema was suspected, but later it seemed probable that all were dependent upon a single etiologic factor, a nematode. Search of the literature has failed to disclose a description of this disease in monkeys. As these animals are commonly used in laboratory experiments, it is thought advisable to make a note of the findings in order that other observers may be saved confusion.

Clinical Description.

The subcutaneous nodules most closely resembling similar nodules in children or adolescents with acute rheumatic fever were found over

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the dorsal aspect of the hands and feet; they were easily missed unless the overlying hair was kept short by frequent shaving. The evolution of a single nodule was as follows: A circular area of subcutaneous edema from 4 to 10 mm. in diameter would appear. Careful palpation at times disclosed a small indistinct nodular thickening in the middle of the edematous area. After 2 or 3 days the edema would disappear and the nodule become more distinct. The smaller nodules, 1 or 2 mm. in diameter, were hard, globular, and definitely subcutaneous, so that the overlying skin could be easily moved over them; often they could be more readily detected by placing the hand or foot of the monkey between a light and the observer, when the overlying skin had a white or gray shining, translucent appearance. Larger nodules were not infrequently ovoid in outline and obviously more easily detected than the smaller ones (Fig. 1). Aside from edema, no objective signs of acute inflammation were grossly manifested in the skin or subcutaneous tissue; sometimes the animals gave evidence of moderate tenderness of the diseased area, at other times they seemed oblivious of any hurt. Following the initial appearance of the nodules they diminished in size so gradually that very little if any difference could be detected from day to day, but measurement every 3 or 4 days demonstrated a definite decrease. The smaller nodules persisted from 1 to 4 weeks; it was often difficult to determine definitely the exact day when they could no longer be detected. Small nodules of this type were seen most commonly over the dorsal aspects of the metacarpal or metatarsal bones, as if closely associated with the tendon sheaths in these regions; several were also seen over the metacarpophalangeal joints; and a few over the proximal phalanges. A few were found over the ulna just below the tip of the olecranon, and over the tendons in the lower third of the leg. Larger nodules, from 3 to 5 mm. in diameter, were occasionally found over the wrist or posterior aspect of the elbow joint. These larger nodules disappeared more slowly than the smaller ones; they never showed evidence of softening in the center.

A second type of subcutaneous lesion was found more frequently over the muscles of the arm (Figs. 2 and 3), forearm, and thigh, less frequently over the leg. It consisted of swellings of irregular size and extent, in which the process seemed to involve both the skin and sub-

cutaneous tissue. At times these papules were flat, from 5 to 10 mm. broad and from 8 to 20 mm. long, and seemed to be from 2 to 3 mm. in depth; at other times they were narrow ridge-like thickenings, 2 to 3 mm. broad and 5 to 30 mm. long, sometimes straight, but more often comma-, L-, or horseshoe-shaped. In some of these lesions indefinite nodules could be detected; in others the swelling assumed a diffuse serpiginous appearance. The nodules, if present, often did not have the definite discrete feeling on palpation that was so characteristic of the lesions over the tendons of the hands and feet. Occasionally, when the lesion was close to a superficially lying bony surface, discrete nodules were very distinct. The more diffuse appearance of many of the papular lesions over muscular areas seemed to be due to a more intimate association between the nodule and overlying skin, and possibly more persistent edema than was present in the lesions over tendon sheaths and joints. Occasionally larger single nodules similar to those about the joints were found over large muscle groups. Rarely, localized areas of firm cutaneous edema from 5 to 10 mm. in diameter were noted; they would persist from 2 to 5 days and quickly disappear without leaving any gross evidence of cellular infiltration. They resembled urticarial wheals more than subcutaneous rheumatic nodules.

A more extensive form of subcutaneous swelling was found about the ankles and wrists of some of the monkeys. It was usually seen in only one extremity at a time, although in one instance both feet were involved simultaneously (Figs. 4 to 7). It consisted of a diffuse thick, soft, edematous area, not sharply outlined; the overlying skin at times was glistening, due to the tension; but there was no other local sign of inflammation. Occasionally the monkey favored the affected limb. At times the edema was confined to the dorsum of the hand or foot (Fig. 8); again it was seen only about the internal or external aspect of the wrist or ankle joint. It lasted from 2 to 10 days. Once or twice the swelling disappeared after 2 or 3 days but reappeared after an interval of 3 to 5 days.

Attempts to obtain fluid by puncture of the swollen area yielded only a small amount of blood-tinged serum. Puncture of the underlying ankle joint in two instances yielded only 1 or 2 drops of normal joint fluid that gave no bacterial growth on ordinary aerobic cultures.

No nodules were found in the subcutaneous tissues following the subsidence of this massive edema; but it was soon found that this type of swelling was always associated with a peculiar blister of the palms or soles in the immediate neighborhood of the swelling. The nature of these blisters gave the clue to the causation of all of the lesions described.

The most obvious pathologic alteration of the palms or soles was an elongated blister 3 to 5 mm. broad, and 2 to 10 cm. long, serpiginous in form as though something had burrowed between the horny epidermis and corium and deposited an irritating substance in its tract. Closer observation of the evolution of the bulla revealed at first a small oval blister about 3 by 5 mm. in size, filled with a blood-tinged serous fluid (Fig. 9); a day or two later it increased to an elongated serpiginous blister filled with similar material (Fig. 10); after another day the fluid became purulent, and the lesions had a yellow color. At this time a portion of the epidermis would be knocked off and the contents of the blister discharged; at times the bulla burst before the fluid had become purulent. In either event, the epidermis over the blister usually desquamated, leaving a smooth elongated serpiginous area with borders composed of ragged skin (Fig. 11). Gradually the denuded areas were covered with normal skin; and after 2 or 3 months the hand or foot showed no evidence of having been diseased. With the exception of the integument at the base and side of the nails, the only cutaneous area that has shown the bullous type of reaction has been the horny skin over the palms and soles, and between the fingers and toes; occasionally a blister was seen at the border of the palms extending 1 or 2 mm. into the neighboring hairy skin.

Occasionally by examination of the palms or soles with a hand lens a very fine serpiginous burrow was seen under the epidermis; it was from 0.3 to 0.5 mm. wide and several centimeters long, gray to brown in color, with no gross evidence of inflammation of the tissue at its side. At times the end of the burrow seemed to communicate with a collapsed blister. It is possible that this fine lesion, appearing as though a thread had been drawn in an irregular direction under the epithelium, is the earliest manifestation of the disease in the palms and soles—a stage before any tissue reaction has occurred.

Examination of the fluid obtained from the blisters revealed a lemon-shaped egg, characteristic of certain nematodes. Later adult worms, the diameter of a hair, about 2 cm. long, were obtained from several lesions.

Examination of the stools failed to reveal either the worm or eggs of this type. Microscopic examination of the tissue removed during life indicated that all of the lesions were due to the presence of a worm.

Temperature.

The temperature of the monkeys varied from 101.5–103.5°F., with an average afternoon temperature of about 1°F. higher than that of the morning. The temperature of the room exerted a certain influence, the temperatures of the monkeys being relatively low when the animal house was cold and high when it was warm.

In general, the presence of blisters and nodules did not influence the temperature of the monkeys. One animal having nodules over long periods, and also inoculated with human joint fluid on two occasions, showed only an occasional rise in temperature above 103°F. In a few instances, when infection of the blisters had occurred, accompanied by swelling and redness of the ankle or wrist, there was fever of 105°F.

Certain of the monkeys, both control and inoculated, at various times had unexplained short febrile bouts.

Blood Picture.

The blood picture resulting from this nematode infection was difficult to determine, as most of the monkeys at this time harbored another nematode of the genus *Æsophagostomum*, this latter being found encysted in the wall of the intestine, mesentery, and omentum. In the monkeys examined, which showed blisters and nodules, the leucocytes varied from 13,000 to 30,000 per c.mm. Leucocyte counts of monkeys not showing such lesions varied from 14,000 to 20,000 per c.mm. Eosinophilia, 7 to 30 per cent, was present in all monkeys examined.

Histopathology of the Subcutaneous Nodules.

Though the various clinical pictures are better understood after a study of the histopathology of the nodules of different ages, it is preferable to give a composite description of the lesions of various ages rather than to describe single nodules. The worm will be described in detail later. In all of the subcutaneous lesions in which it was found, it was from 35 to 55 microns in diameter and delimited by a hyaline cuticle staining pink with eosin-methylene blue, red with Van Gieson, and pale red to grayish red with methyl green-pyronine. This cuticle was at times the only worm structure that could be detected in older nodules; it seemed to be the portion of the parasite most resistant to the defensive agencies of the host.

In the youngest lesion, in which very little except edema of the skin and underlying tissues was clinically demonstrable, the area upon removal had a diffuse, gray, moist appearance, and no definite nodule could be detected. Microscopic examination of such an area usually revealed a single cross-section of the worm in each section; the parasite was surrounded by a collar of epithelioid cells and polymorphonuclear eosinophils; the total thickness of the collar was from two to five cells (Figs. 12 and 13). Small blood vessels nearby showed a marked perivascular reaction made up of actively dividing epithelioid cells (endothelial leucocytes) and polymorphonuclear eosinophils; vessels situated at least 10 mm. from the worm often showed this perivascular reaction (Fig. 14). The surrounding connective tissue showed distinct edema with scattered eosinophils (Figs. 12 and 13). In some slightly older lesions the worm and collar of cells were surrounded by a narrow circular area filled with fibrin; this in turn was encompassed by an area made up of a loose network of fibrin and cells with branching cell bodies and pale vesicular nuclei (Fig. 14). Edema, perivascular reaction, and diffuse infiltration with eosinophils were present in the surrounding tissue of all of the early lesions. In an area slightly older than the one just described many newly formed thin walled blood vessels were seen; also a few polynuclear cells with two, four, eight, or more closely packed, pale vesicular nuclei and very little cell body; they were probably young giant cells.

In those lesions, however, in which a distinct nodule from 2 to 4 mm. in diameter was evident at the time of earliest observation, microscopic examination revealed several coils and cross-sections of the parasite in each section; it seemed as though the worm were confined to a smaller area with consequently a more intense local reaction (Figs. 15 and 16). Some coils of the worm were surrounded simply by coagulated blood, others by a collar of cells similar to that previously described; often many of these cells had deep staining pycnotic nuclei as if they had been injured by some toxic agent; still other sections of the parasite were surrounded by epithelioid cells, lymphocytes, eosinophils, and giant cells. In two nodules this area of worm, hemorrhage, and cell reaction was surrounded on all except one side, or peduncle, by a zone of fibrillar and granular material into which were growing many young actively dividing cells with pale, vesicular nuclei and cell bodies with long branching processes; these young cells could be seen arising from the connective tissue surrounding the nodule. This area of actively growing cells closely resembled the edge of a tissue culture (Fig. 16). The portion of the lesion called the peduncle was made up of many newly formed capillaries and cells with large, pale, vesicular nuclei. As one passed towards the normal connective tissue these cells approximated more and more the appearance of the fixed connective tissue elements. In none of the lesions of this age was there much capsule formation, although in some areas a mosaic of spindle cells with large pale nuclei could be detected; methyl green-pyronine stained sections showed plasma cells most numerous in this zone. Practically all of the small blood vessels within a radius of 3 to 5 mm. showed the marked perivascular reaction previously mentioned; about some of them many closely packed plasma cells were visible, and about others were numerous eosinophils. In fact, eosinophils were present throughout all portions of the sections, both in the zone of active cell proliferation and in the surrounding edematous tissue.

In a slightly older lesion the coils of worm were immediately surrounded by mononuclear cells in many of which the nuclei were deep staining and pycnotic; next to this zone there were a number of large, giant cells mixed with many eosinophils and a moderate number of epithelioid cells (Figs. 17 and 18). Scattered throughout the nodule

were several areas made up largely of newly formed capillaries distended with blood. At the periphery of the nodule definite early capsule formation was present; outside of this the blood vessels still showed marked perivascular reaction, and small foci of edema were seen in the connective tissue in contradistinction to the diffuse edema seen in the earlier lesions. A nodule having approximately the same general structure showed in only eleven of the sections easily distinguishable cross-sections of the parasite; nearby were annular hyaline structures of the same thickness, diameter, and staining reaction as the cuticle of the worm; occasionally these rings were filled with mononuclear wandering cells. No large giant cells were seen in this lesion, but there were several smaller cells with eight to fifteen closely packed oval or round vesicular nuclei and a distinct cell body of a red, finely granular appearance in methyl green-pyronine stained sections. These cells closely resembled the large cells seen in the Aschoff bodies in the hearts of patients dying in the acute stages of rheumatic fever. In fact, in this nodule it would have been difficult to differentiate the histological structure from that of early subcutaneous nodules in children with rheumatic fever, had no sections of the worm been found.

In nodules 2 weeks or more old capsule formation was more marked, many plasma cells were found immediately under the capsule, and young actively dividing cells were comparatively less numerous in the interior of the lesion. Distinct cross-sections of worms were found in only three out of seven old nodules examined; and in these only a few of the sections contained a single cross-section of worm. In one other lesion hyaline ring-like structures resembling the cuticular membrane were seen. In all of the older lesions containing a portion of the worm there were many large giant cells often surrounding the parasite like a ring; in nodules containing no worm they were less numerous and were absent in two nodules with very thick capsules (Fig. 19). It seems probable that these giant cells function to remove the parasite and disappear as soon as this task is completed; this view is substantiated by their being most numerous in the nodules in which the worm is undergoing disintegration and by their gradual disappearance in lesions in which no remains of the parasite can be found. The presence in the younger lesion of polynuclear cells with two, four,

eight, or more nuclei and the fact that these cells are not far from the worm suggest that the giant cells are formed from the young actively growing cells that have invaded the lesion. A study of many lesions of various ages revealed this giant cell development from cells containing two nuclei to old cells with many nuclei peripherally situated.

Nodules a month or more old consisted chiefly of fibrous tissue in which the cells were more densely arranged at the periphery (Fig. 19). Eventually these nodules completely disappear, for none could be found post mortem in several monkeys killed 3 or 4 months after the clinical disappearance of multiple subcutaneous lesions.¹

Histopathology of the Palmar and Plantar Lesions.

The chief site of activity of the nematode in the palms and soles was in the epidermis, with only slight accompanying reaction in the superficial portion of the corium. The principal portion of the epidermis involved was the stratum mucosum below the stratum lucidum and above the stratum germinativum. An early lesion taken from a place where the worm was grossly detected, and cut so that several cross-sections of the parasite were present in a single section, showed different degrees of reaction. Occasionally a single cross-section of the worm was seen in the stratum mucosum of the epidermis with the neighboring epithelial cells appearing almost normal. In other places, close by, small areas slightly larger than the diameter of the parasite were seen filled with granular material, red blood cells, and degenerated epithelial cells (Fig. 20). Generally the area of reaction about the parasite was much larger and consisted

¹ Incidentally another type of subcutaneous nodule was found in several monkeys, most often about the extensor aspect of the elbows. Grossly they were 1 or 2 mm. in diameter and consisted of a small brown central portion surrounded by a tough white membrane. Microscopically the central portion was made up of six-sided cells with a thick membrane containing a more or less homogeneous material; the entire cell stained deep blue with methylene blue-eosin. They were thought to be vegetable cells making up possibly the points of thorns or splinters of wood. Surrounding the mass of vegetable cells were a few foreign body giant cells and a dense fibrous tissue capsule. Nodules of this type remained constant in size and never showed the perinodular and subcutaneous edema noted about the lesions containing a worm.

in cross-section of a vesicular area 1 mm. thick and 2 to 4 mm. in breadth. In early lesions before rupture of the blister, the worm was usually found close to the stratum germinativum, which in most places seemed to be intact, but occasionally small areas of this stratum were missing and the blister was in direct contact with the superficial layers of the corium. In these places it was not unusual to see capillaries coursing upward through the corium and emptying into the blister. In the superficial layers of the corium immediately under the blisters there was a definite increase of cells, usually mononuclear in type, but in places small groups of polymorphonuclear leucocytes were seen. Rarely did the small blood vessels of the corium show the perivascular reaction which was so prominent a feature in the subcutaneous nodules. Occasionally there was definite edema of the subcutaneous tissue surrounding the blister, with a moderate number of polymorphonuclear eosinophils scattered through the edematous area.

The contents of the early unbroken blister consisted of amorphous granular material, many red blood cells, and a few leucocytes, both mononuclears and polymorphonuclears; epithelial cells in various stages of degeneration were also present. In older blisters there were many polymorphonuclear leucocytes but fewer red blood cells and epithelial cells. In most of the unbroken blisters it was possible to find the characteristic eggs of the nematode, which in all instances contained fully developed embryos. At times, surrounding the eggs there was a thin, pink-staining, hyaline, annular structure, irregular in outline and two or three times the diameter of the egg. Some sections of the blisters contained the adult parasite, others did not; if present the number of cross-sections of the worm in a single section varied from one to six or eight. In some lesions excised following an unsuccessful attempt to remove the worm intact, small portions of the parasite were found winding through the epidermis. This peculiar winding made it difficult to obtain many complete uninjured specimens of the nematode from the blisters. Sections of skin removed after the blister had ruptured showed the stratum corneum and stratum lucidum separated from the stratum mucosum by an open space; usually the stratum germinativum was intact but occasionally it was missing for a short distance. The fact that the deepest layer of

epithelium was rarely completely destroyed and that there was little destruction of the superficial layers of the corium explains the usual absence of deep ulceration after the desquamation of the top of the blisters; the germinal layer of the epithelium was in condition quickly to repair the damage.

It is evident that the two types of lesions differ in more than one respect. In the subcutaneous nodule the nematode is smaller in diameter, and probably in length, although this point cannot be definitely determined because of the impossibility of obtaining specimens of the parasite except in sections from the subcutaneous tissue. The anatomy of the worm found in the nodules was much simpler than that of the female nematode found in the cutaneous blisters (Figs. 21 to 23). No bodies resembling eggs were ever seen in the worms in the subcutaneous lesions or in the tissue immediately surrounding them; on the other hand, eggs in various stages of development were found in all of the parasites in the epidermal blister, and eggs containing embryos were constantly present in the blisters. In both the subcutaneous lesion and the epidermis the first effect of the parasite seemed to be necrosis of the cells immediately surrounding it, followed by vesicle formation with a rapid filling of the vesicle by red blood cells.

The hemorrhage surrounding the female worm in the epidermal burrows and many of the worms in the subcutaneous tissue is probably due to some toxic substance secreted by this parasite. Schwartz (1) lately showed that certain nematodes contain substances that inhibit the coagulation of blood to a marked degree and appear to be similar to leech extract. This toxic substance is evidently of importance in insuring the worm a proper supply of food, for the adult female lives literally in a pool of blood. The finding of hemorrhage about many of the nematodes in the subcutaneous nodules is a point in favor of the theory that the worms in both the epidermis and subcutaneous nodules belong to the same species.

In the subcutaneous tissue the intensity of the local response was so great that the parasite was prevented from wandering farther; on the contrary it seems to have been quickly killed, firmly encapsulated, and subsequently removed like any other easily phagocytatable foreign body. In other words, the response of the tissues of the host in this re-

gion was sufficient to prevent the parasite from continuing its existence. The conditions in the epidermal lesion, on the other hand, were more favorable for the parasite. By continuing its existence in the epidermis during the egg-laying stage and constantly invading new skin areas, the female worm found favorable conditions for the deposition of its eggs and their extrusion into the external world where they might infect new hosts. The reaction in the epidermis never seemed to be sufficiently intense to kill the worm. On the contrary, the ease with which the blister burst and scattered its contents rendered especially easy the dissemination of the eggs. There is here a condition of almost perfect parasitism in which the injury inflicted by the nematode on the host is not sufficient to impair seriously the health of the host, but in which the local injury in the palms and soles is of such a nature as to favor the continuation of the existence of this peculiar nematode.

It has been impossible for us to determine the life history of this nematode. It is possible that some of the worms found in the subcutaneous lesions were males; but if they were males, one cannot state whether they migrated to the subcutaneous tissue after fertilizing the female or whether they reached this tissue before having completed their sexual function. We have examined post mortem several monkeys with active skin lesions and, with the exception of one lymph node containing a larval form and tubercle-like nodule, have found no lesions resembling the skin blisters or subcutaneous nodules in any of the viscera or muscles. In so far as our observations go they indicate that practically the entire lesion producing activity of this nematode is in the epidermis and subcutaneous tissue.

Attempts to hatch the eggs in moistened filter paper kept at body temperature for 2 months have failed to induce the embryos to migrate from the shells. The injection of the eggs into the skin of the palms and soles of monkeys and into the subcutaneous tissue of the extremities has not been followed by lesions like those observed in naturally infected animals; there was slight local reaction lasting 3 or 4 days, following which there was practically no gross evidence of the introduction of any irritating substance. The fact that fully developed embryos can be caused to emerge from the eggs obtained from skin blisters by simple mechanical pressure shows that they are probably in condition to continue their life's activity

immediately when brought under favorable conditions. It seems most probable that the digestive juices of the stomach or intestine act upon the opercular plug and allow the embryo to escape; it is well known that eggs of this character are usually hatched in the stomach or intestine, after which the embryos continue their activity by burrowing through the wall of the gastrointestinal tract and migrating to various parts of the body. An intermediary host is therefore not necessary for the continuation of this type of parasite.

We have searched in vain for the characteristic eggs in the feces of twenty monkeys that had shown skin lesions and in the entire intestinal contents removed post mortem from several other monkeys; Kofoid's (2) brine concentration method was employed to obtain the material for examination. The eggs of another common intestinal parasite, an *Æsophagostomum*, were found in the feces of most of the monkeys examined. It seems probable, therefore, that the only manner in which the *Trichosoma* eggs are extruded from the body of the monkeys is from the skin lesions. In so far as we are able to determine, this method of dissemination of eggs has not been previously described for nematodes.

It is not unusual to find lesions of the skin and subcutaneous tissue caused by animal parasites. Ground-itch is well known to be due to invasion of the skin by the larvæ of *Ancylostoma duodenale*. Looss (3) states that a similar condition may result from infection with larvæ of *Strongyloides stercoralis*. Subcutaneous tumors in man may be caused by *Filaria bancrofti*, *Loa loa*, *Agamofilaria georgiana*, and *Rhabditis niellyi*. The female guinea-worm (*Dracunculus medinensis*) burrows in the subcutaneous tissues and discharges young larvæ from her body, through an ulcer in the skin to the outer world. This is the closest parallel we can find in the literature to the action of our *Trichosoma* in which eggs are deposited in an epidermal blister before being discharged from the body of the host.

A somewhat similar microscopic lesion of the epidermis is seen in larva migrans, or creeping eruption, which is due to burrowing in the epidermis by the larvæ of a *Gastrophilus* (bot-fly). The gross picture, however, is different; it consists of a migratory red line in the skin from 1 to 9 mm. broad, in the center of which is a fine line due to the burrow. Minute vesicles are occasionally seen along the course of

the red line, but the entire lesion does not become bullous or purulent (4).

Castellani and Chalmers (5) report a rare skin disease of unknown etiology found in natives of Ceylon and South India; it is called dermatitis macrogyrata, and is characterized by the presence on the palms of the hands of one or two very large gyrations formed by scaling and crusty lesions. On removing the crusts and scales a broad shallow furrow can be seen situated in the epidermis. Neither fly larvæ nor fungi are found in the lesions. The photograph of the condition in Castellani and Chalmers' book resembles very closely the lesions seen in our monkeys after the removal of the top of the blister. It is interesting to conjecture upon the possibility of the two conditions being due to a common cause, inasmuch as *Macacus rhesus* comes from the same part of the world as the patients in whom dermatitis macrogyrata is found.

Description of the Nematode.

Female.—Only a few intact specimens of the female worm were obtained from the blisters on the hands or feet. Many broken specimens were secured, all of which contained eggs. The worms were all of practically the same size; the head and esophagus were of about one-half the diameter of the portion of the body containing eggs; the body gradually increased in diameter from immediately behind the head to the tail. Measurements were as follows:

Head: Length 42 microns; width 52 microns.

Diameter of worm at posterior end of esophagus: 100 to 110 microns.

Diameter of posterior portion of body: 200 microns.

Length of body: 22 to 24 mm.

Esophageal portion of body: One-sixth of the entire length of the worm.

In unstained specimens fixed in alcohol and mounted in glycerol, the nematode showed a hyaline cuticle with slight annular striation. Beginning about the middle of the body were seen numerous highly refractile fine papillæ, arranged in two broad bands towards the posterior portion of the body. The cuticle about the head was about twice the thickness of that elsewhere; the head was thus slightly ovoid in shape. No teeth could be detected. The esophagus con-

sisted of a single chain of from 85 to 95 cells with the lumen in the center. The digestive tube then showed a slight sacular dilatation, followed by a long intestine which was arranged in loose coils in the posterior half of the body. The anus was terminal. The posterior end of the body was bluntly conical.

The vulva was situated just posterior to the termination of the cuboidal esophageal cells. There was a single ovary at the posterior end of the body. Between the ovary and the vulva the eggs could be seen in the uterus gradually developing into definite embryos and taking on the characteristic brown, lemon-shaped shell with round canal at each end covered by an opercular plug.

The eggs measured from 40 to 42 microns in breadth and 67 to 70 microns in length, had a thick highly refractile hyaline shell, usually brown in color, and contained a coiled embryo, which upon pressure could be caused to emerge through the opening at one end (Fig. 24).

The embryo had bluntly rounded ends and was filled with a uniform colorless material containing a group of highly refractile droplets toward the posterior end.

In sections of the epidermis stained with eosin-methylene blue, the female parasite was surrounded by a pink hyaline cuticle. Inside of this were two single rows of blue-staining cuboidal muscle cells; each row occupied about one-third the circumference of the worm (Figs. 25 and 26). Between these rows of cells and slightly overlapping the ends were more loosely arranged pink-staining cells with long communicating processes, considered as nerve cells. In all cross-sections a flattened annular structure, evidently the intestine, was seen lined with a single layer of cuboidal cells. In a number of instances the lumen of the intestine contained a few erythrocytes.

In some sections the remainder of the body was filled with a single cross-section of the uterus with maturing eggs (Fig. 26). Elsewhere, there were two or three cross-sections of the generative organs. Some contained young eggs, others consisted of a thin hyaline ring enclosing numerous long flat deep blue-staining bodies radially arranged and usually pointing towards the center. Mesial to these, there was a loose fibrillar meshwork with a few similar elongated bodies. In a few sections large cells resembling young eggs were seen in the

center of this organ, and in several longitudinal sections this organ (supposed to be the receptaculum seminis) was seen to communicate directly with the egg-containing structure (Figs. 25 and 27).

No sections were obtained through the esophagus of the female worm. In all of the sections above described the diameter of the worm was from 140 to 160 microns.

Worm in Subcutaneous Nodules.—In one series of sections the entire worm was included (Fig. 28). It was fairly uniform in size, and measured from 35 to 45 microns in diameter. It was surrounded by a thin hyaline pink-staining cuticle, inside of which was a single ring of cuboidal cells. In some places this ring of cells was almost continuous; elsewhere, it was broken into two segments each occupying about one-third the circumference of the worm, with one or two larger cells between the ends of the segments. In many cross-sections the remainder of the worm was filled with granular amorphous pink-staining material. On longitudinal section, one end of the worm was made up of cuboidal cells filling the entire parasite. These resembled the esophageal cells seen in the female, and evidently corresponded to the same pink-staining areas described in cross-sections. In other sections, most often towards the posterior end of the worm, were seen two circular structures made up of elongated flat deep blue-staining bodies similar in shape and size to those seen in the receptaculum seminis of the female worm. In longitudinal sections these flattened bodies were pointed antero-posteriorly. No lumen or structure resembling an intestine was seen in this specimen.

A fresh serpiginous lesion after excision showed on gross examination a small waxy line. In these sections many cross-sections of a worm were seen; they were larger and the worm was evidently more mature than the one described above; measurements of several cross-sections showed a diameter of about 55 microns (Figs. 29 to 31). There was a pink hyaline cuticle inclosing a single ring of cells. This ring was divided into four segments, two of which contained cells with rounded deep blue-staining nuclei, each segment occupying about one-third the circumference of the worm; the other two were made up of pink-staining cells resembling the nerve cells of the female worm. Back of the esophagus were two distinct structures in the body of the

worm. One was an elliptical ring lined with a single layer of flat cells, evidently the intestine. The other was a round body occupying about one-half the diameter of the worm and in places filled with small cells with solid deep blue nuclei; in other places the nuclei were arranged concentrically about a small lumen; in still other sections the lumen was larger and surrounded by a single layer of cuboidal cells. At the head end of the worm longitudinal sections showed large cuboidal esophageal cells, and cross-sections showed these cells occupying the entire diameter of the parasite inside of the muscle and nerve cells.

It was thought probable that this was a male worm. The muscle and nerve cells, esophageal cells, and intestine resembled similar structures of gravid female worms in the epidermis. The genital tract, however, was different.

The first of the worms described in the subcutaneous tissue was evidently a larval form, as neither the intestinal nor genital tract was well differentiated. The second was more mature, with a genital tract distinctly different from that of the adult female. Both forms were seen in other sections. It seems plausible, therefore, to conclude that the subcutaneous nodules contained both immature larvæ and fairly well developed male worms.

Classification of the Nematode.

The parasite is clearly a member of the nematode superfamily Trichinelloidea Hall, 1896a, family Trichinellidæ Stiles and Crane, 1910. Hall (6) divides this family as follows:

- "1. Male with 1 spicule or at least with a copulatory sheathTrichurinæ.
"Male without spicule or copulatory sheath.....2.
- "2. Eggs spherical without true egg shell; ovoviviparous; males not parasitic in females; adult worms in digestive tract.....Trichinellinæ.
"Eggs variable in shape and with true, thick shell and opercular plugs; oviparous; males parasitic in the uterus of females; worms in kidney pelvis ureters or in the urinary bladder.....Trichosomoidinæ."

On characters of the female, namely size and shape of body and location in host, our worm can be safely eliminated from the sub-family Trichinellinæ. As an examination of ten gravid females failed

to reveal the presence of males in the uterus, this worm is apparently to be eliminated from the subfamily Trichosomoidinæ. Hall (1916a, p. 19) divides the remaining subfamily, Trichurinae, as follows:

- "1. Anterior esophageal region of body very slender and longer than the posterior portion, which is much thicker and contains the reproductive organs.....*Trichuris*.
 "Anterior esophageal portion of body shorter than, rarely equal to, the posterior portion, which is only slightly thicker.....2.
- "2. Worms with spicule; in digestive tract or urinary bladder, ducts, etc.
*Capillaria*.
 "Worms without spicule; in liver.....*Hepaticola*."

This key clearly eliminates the genus *Trichuris* from consideration. There remain for consideration the following closely allied genera: *Capillaria* Zeder, 1800a, and *Hepaticola* Hall, 1916a. *Capillaria*, s.l. (synonym *Trichosoma*) is divided by various authors into *Capillaria*, s. str., *Thominx* Dujardin, 1845a, and *Calodium* Dujardin, 1845a, which are variously recognized as genera or as subgenera.

It will be noticed that to classify a species of Trichurinae in its proper genus and subgenus, it is absolutely essential to examine the male worm, and this sex we have thus far been unable to find. Any generic classification is, therefore, only provisional and will of necessity be subject to possible change as soon as the male is studied.

The old collective genus of this group is *Trichosoma* Rudolphi, 1819a, which is *Capillaria* Zeder, 1800a, renamed. If we classify the worm in *Capillaria*, s. str., *Hepaticola*, *Thominx*, or *Calodium*, it is clear that we predicate certain anatomical characters upon the basis of which the modern classification is founded; accordingly, we introduce an element of confusion in anatomy. By placing the worm in the collective genus *Trichosoma* (which is *Capillaria sensu lato*) we avoid all complications as to detailed points in anatomy, but make it inevitable that later the worm will be transferred to one of the modern and more restricted generic groups. Both courses are open to criticism, but it seems that the latter course is the more conservative, since changes of names are more easily made than changes in erroneous conceptions as to anatomy; further, *Trichosoma* gives the approximate, though not the taxonomically exact, location of the worm. Accordingly, the parasite is provisionally referred to *Trichosoma* pending the finding of the male.

As it has been impossible to find any reference to a dermal trichosome in monkeys, we assume that the species is new and we propose for it the name *Trichosoma cutaneum*, 1922.²

SUMMARY.

A number of monkeys (*Macacus rhesus*) were found to be infected with a nematode which gave rise to several types of skin lesions, subcutaneous nodules, edema about the joints, and elongated serpiginous blisters of the palms and soles.

In the subcutaneous nodules were found larval forms of the nematode and possibly adult male forms.

The reaction about these worms consisted of proliferation of fixed cells, and invasion of eosinophils, with subsequent presence of giant cells, young blood vessels, and finally capsule formation; eventually the worms were killed, eliminated, and the nodule disappeared.

In the skin of the palms and soles the adult female worm burrowed in the epidermis, producing an elongated serpiginous blood blister that eventually became purulent. In this blister the worm laid her eggs; and by the bursting of the blister the eggs were discharged into the outer world and placed in a position to infect new hosts. The reaction in the epidermis was evidently not severe enough to interfere seriously with the health of the host or with the continuation of the egg-bearing period of the female parasite. This condition of almost perfect parasitism is an ideal one for the continuation of the life of this species of nematode. In so far as we are able to determine this is the first description of a nematode that lays its eggs in the epidermis.

The provisional name of the parasite is *Trichosoma cutaneum*, 1922.

² In seeking to identify this parasite in literature we have consulted the Stiles and Hassall nematode catalogue (Stiles, C. W., and Hassall, A., *Bull. Hyg. Lab., U.S.P.H., No. 114, 1920*), Stossich (Stossich, M., *Boll. Soc. adriat. sc. nat. Trieste*, 1890, xii, 3), Travassos (Travassos, L., *Mem. Inst. Oswaldo Cruz*, 1915, vii, 146), and various other publications, but have found no record of cutaneous *Capillaria* in monkeys; neither has a record of this kind been found in the host catalogues of parasites in the Zoological Division of the United States Public Health Service and of the Bureau of Animal Industry.

For valuable assistance in this portion of the work and many helpful suggestions we are indebted to Professor Charles Wardell Stiles of the United States Public Health Service.

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EXPLANATION OF PLATES.

PLATE 39.

FIG. 1. Showing large and small subcutaneous nodules in the dorsum of the hand.

FIG. 2. Showing an elongated papular area above the left elbow.

FIG. 3. Showing a broad papule over the tendon of the triceps.

PLATE 40.

FIGS. 4 and 5. Showing simultaneous swelling of the right ankle and left foot.

PLATE 41.

FIGS. 6 and 7. Lateral view of the ankle and foot shown in Figs. 4 and 5.

PLATE 42.

FIG. 8. Showing pitted edema of the dorsum of the left hand.

FIG. 9. Showing a very early small blister.

FIG. 10. Showing an elongated blister at the base of the great toe.

PLATE 43.

FIG. 11. Showing ragged edged desquamating areas over the plantar region.

PLATE 44.

FIG. 12. Early subcutaneous lesion showing a slight collar of cells about the worm and marked infiltration of eosinophils in the surrounding edematous tissue. $\times 230$.

FIG. 13. Early subcutaneous lesion showing a slightly more marked collar of cells about the parasite and eosinophils in the neighboring edematous tissue. $\times 450$.

PLATE 45.

FIG. 14. Early subcutaneous lesion showing the worm surrounded by fibrin and a few cells, in a tissue space, and perivascular reaction at some distance from the worm. $\times 55$.

FIG. 15. Older subcutaneous nodule containing two cross-sections of a worm. *W*, worm (two cross-sections) surrounded by an intense cellular reaction; *PV*, perivascular reaction. $\times 85$.

PLATE 46.

FIG. 16. Large globular subcutaneous nodule containing many sections of a worm, surrounded by a hemorrhagic zone. *HZ*, hemorrhagic zone; *CZ*, clear zone containing granular material and a few cells; *Y*, zone of young actively dividing cells; *P*, pedicle of the nodule containing many newly formed blood vessels; *PV*, perivascular reaction. $\times 85$.

PLATE 47.

FIG. 17. Older subcutaneous nodule containing a single cross-section of a poorly staining worm. *W*, worm; *G*, giant cells surrounding worm; *C*, capsule; *PV*, perivascular reaction. $\times 85$.

FIG. 18. Higher power of the portion of Fig. 17 showing the worm. *W*, worm; *G*, giant cells. $\times 450$.

PLATE 48.

FIG. 19. Old subcutaneous nodule. $\times 85$.

PLATE 49.

FIG. 20. Low power section of an early unbroken plantar blister containing a female nematode. *W*, worm; *WI*, cross-section of worm with very little necrosis of cells; *V*, microscopic vesicle containing only cell detritus. $\times 48$.

PLATE 50.

FIG. 21. Unstained specimen of female *Trichosoma cutaneum* obtained from a palmar blister. *H*, head; *P*, posterior portion of body; *V*, vulva. \times about 9.5.

FIG. 22. Head and esophagus. *H*, head; *E*, esophagus surrounded by cuboidal cells; *V*, vulva, from which eggs containing embryos are being expelled. $\times 37$.

FIG. 23. Posterior end of the body containing the ovary and young ova. $\times 37$.

PLATE 51.

FIG. 24. Eggs of *Trichosoma cutaneum*, each containing a well developed embryo. $\times 400$.

FIGS. 25 and 26. Cross-section of a female *Trichosoma cutaneum* from a palmar blister. *M*, muscle cells; *N*, nerve cells; *I*, intestine; *O*, ovary; *U*, uterus containing partially matured eggs; *RS*, receptaculum seminis; *E*, empty egg-shell. $\times 410$.

FIG. 27. Longitudinal section of a female parasite from a palmar blister. *RSO*, union of receptaculum seminis and ovary; *RS*, receptaculum seminis; *O*, ovary. $\times 400$.

PLATE 52.

FIG. 28. Young worm in a subcutaneous nodule. *E*, esophageal cells, longitudinal section; *F*, cross-section of esophageal cells; *C*, cross-section of the posterior portion of the body. $\times 450$.

FIGS. 29 to 31. Male (?) form of nematode in a subcutaneous nodule.

FIG. 29. Esophageal cells (*E*), longitudinal section. $\times 450$.

FIG. 30. *E*, esophageal cell, cross-section; *I*, intestine; *G*, genital tract containing lumen. $\times 450$.

FIG. 31. *M*, muscle cells; *N*, nerve cells; *I*, intestine; *G*, genital tract (testis?). $\times 450$.



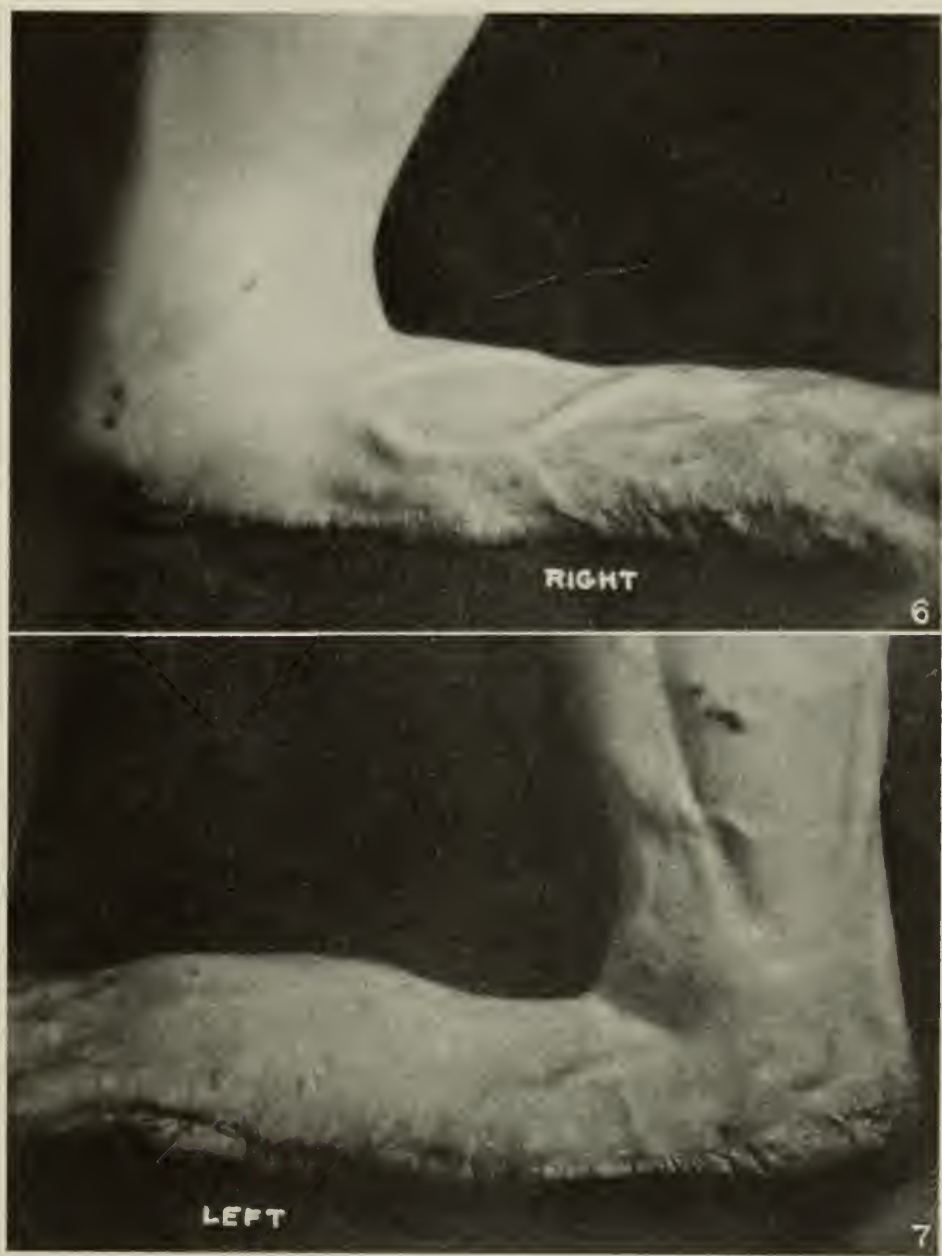
(Swift, Boots, and Miller: Cutaneous nematode infection in monkeys.)

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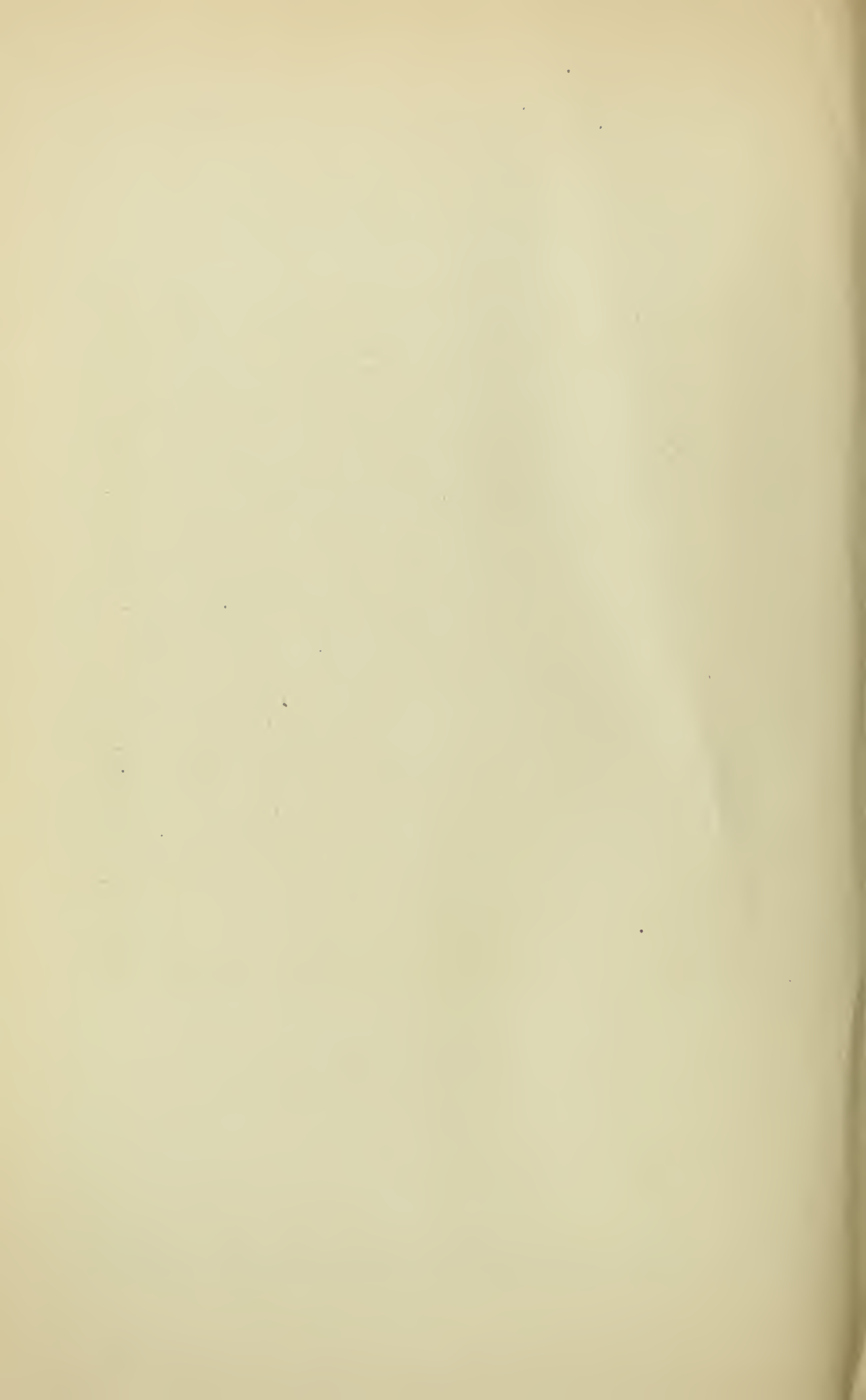


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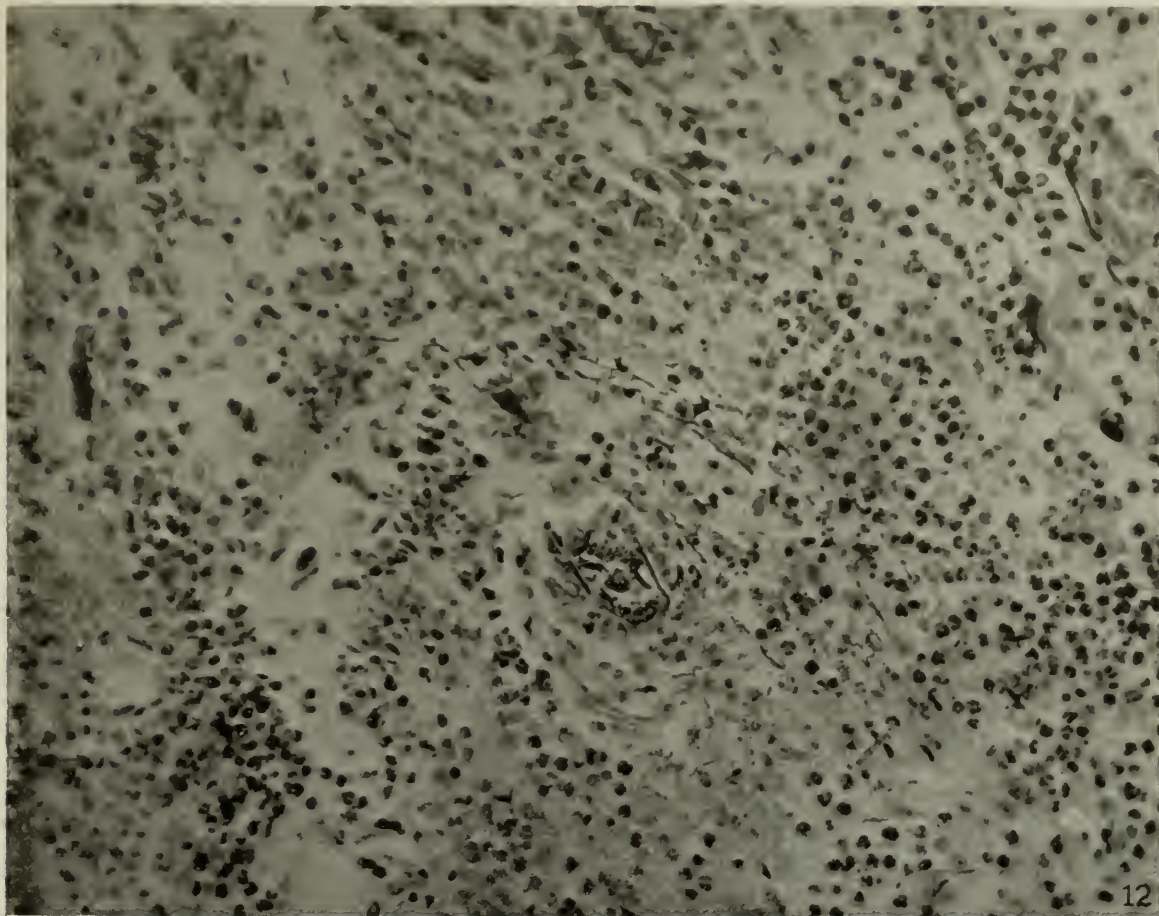


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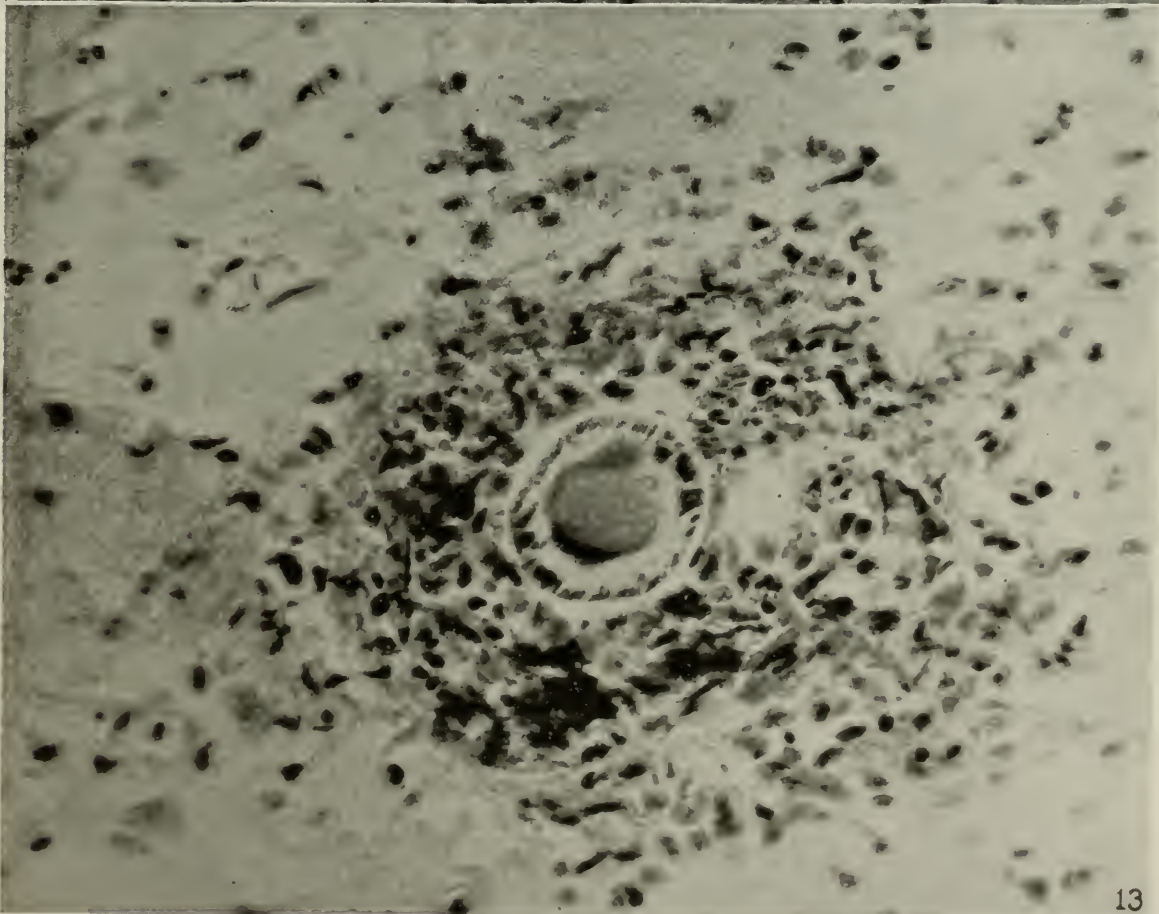


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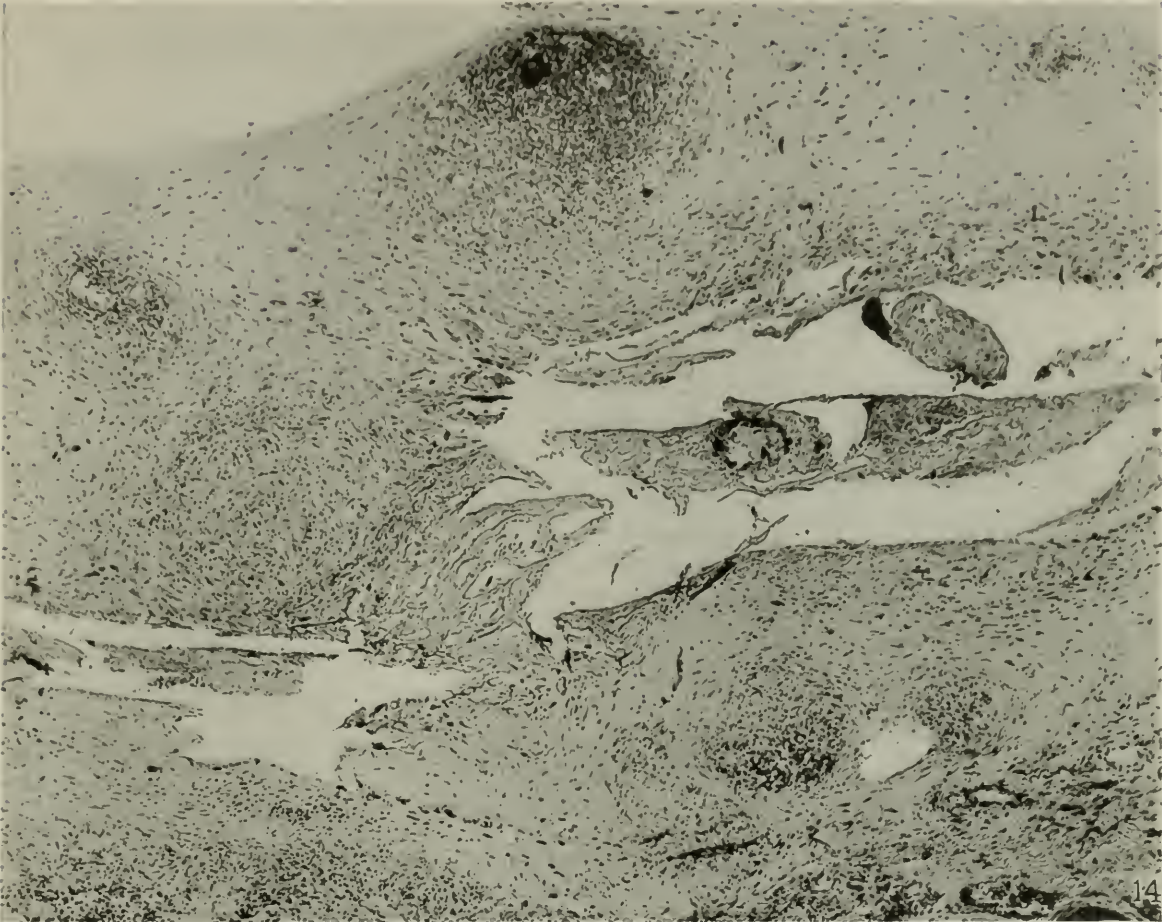
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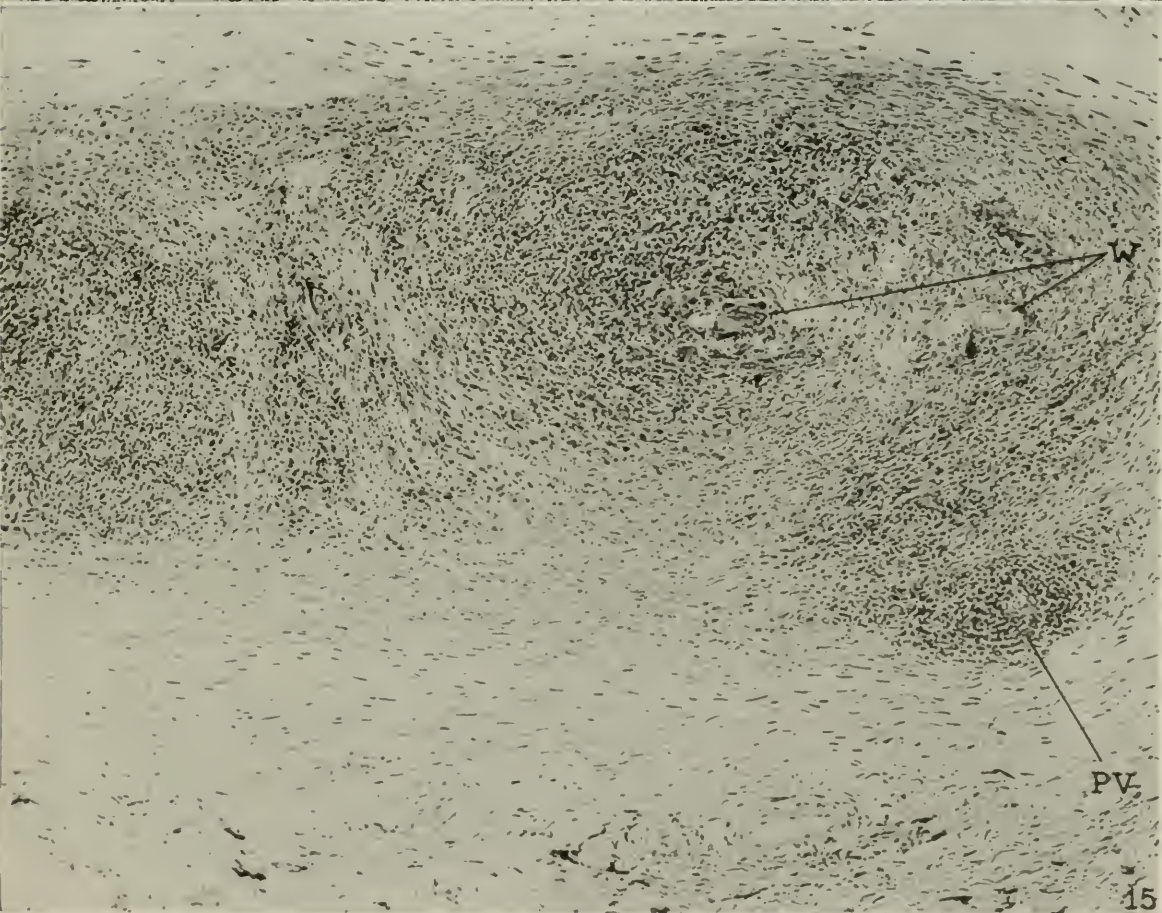
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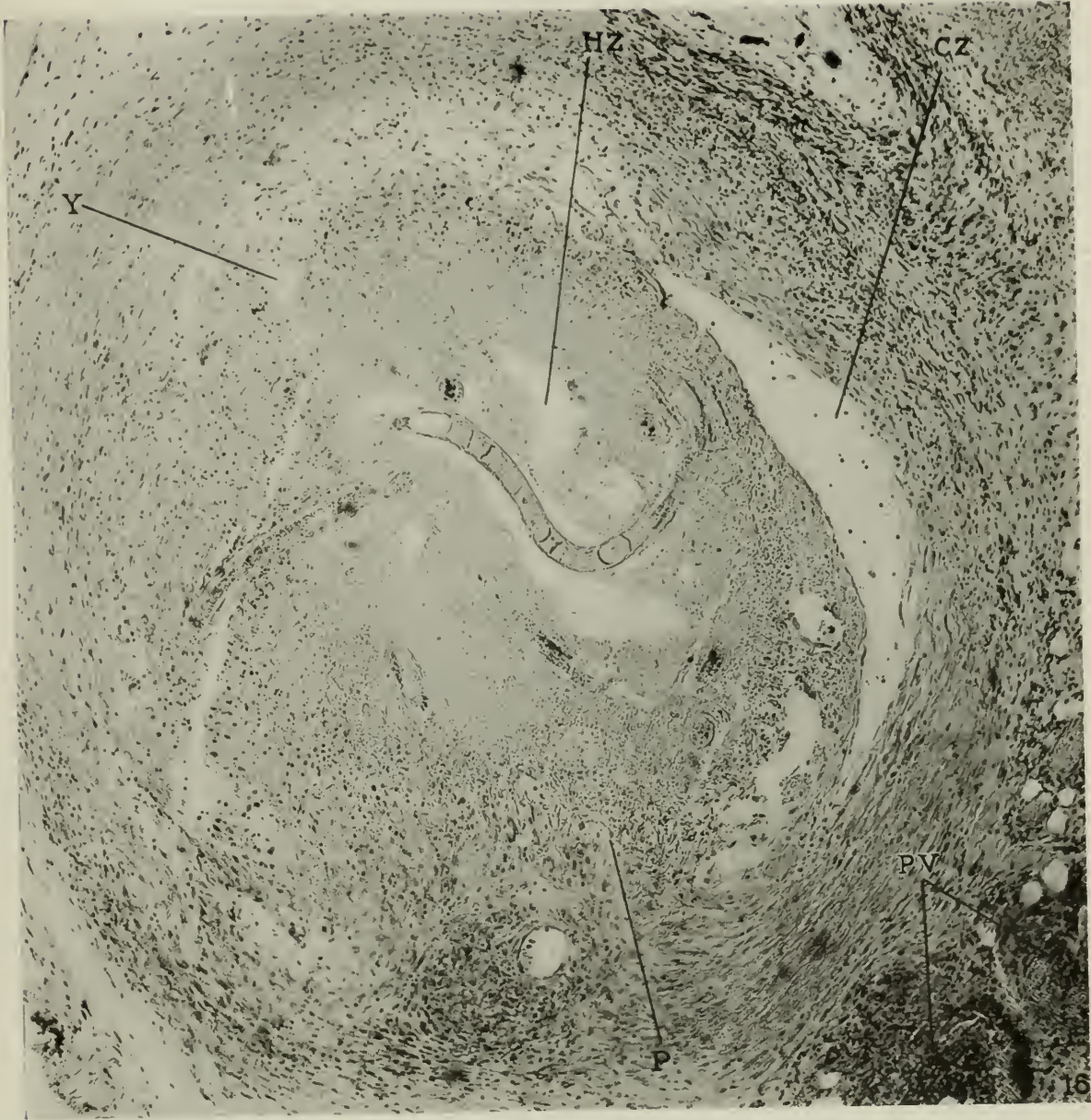
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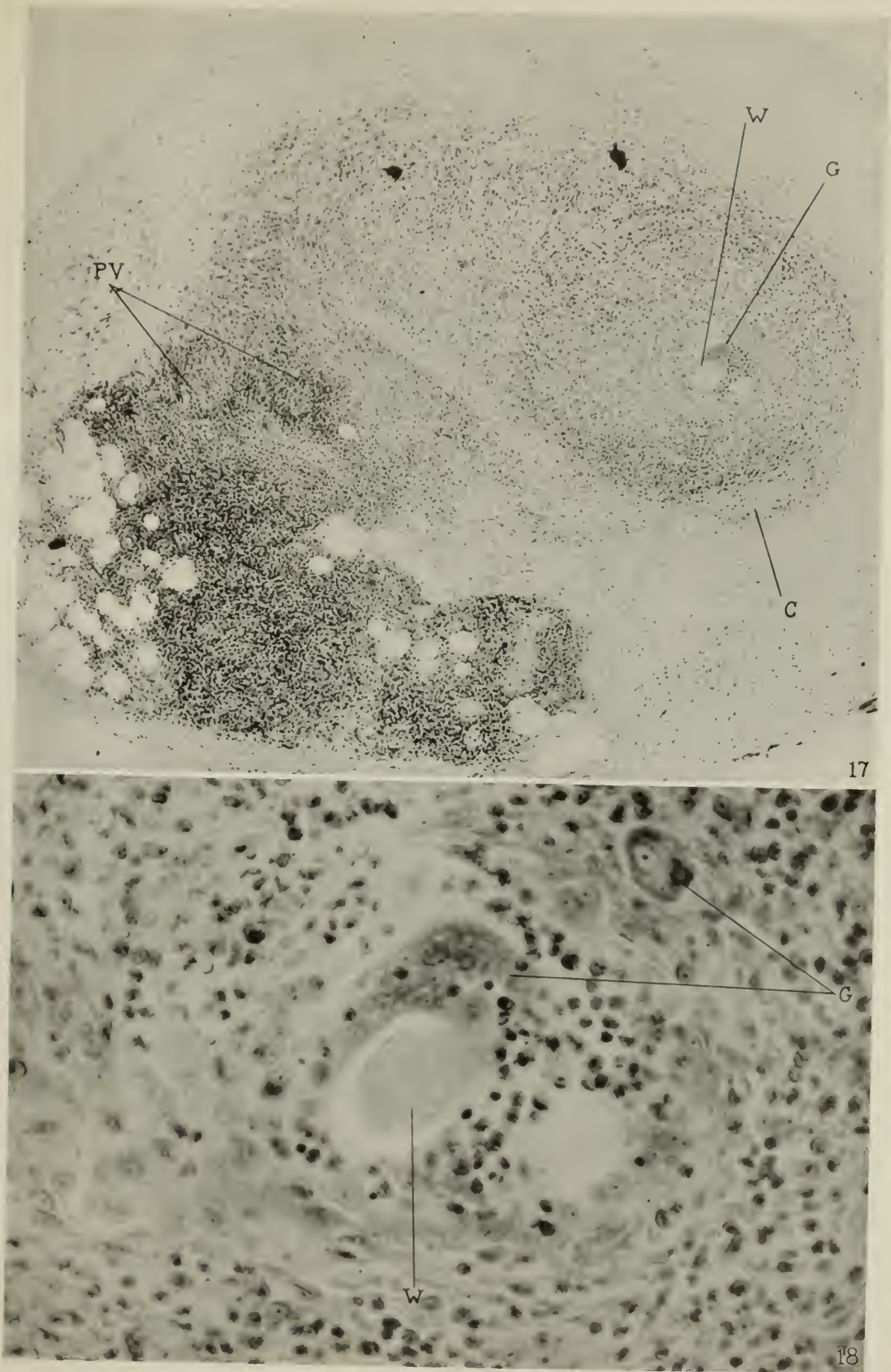
PV

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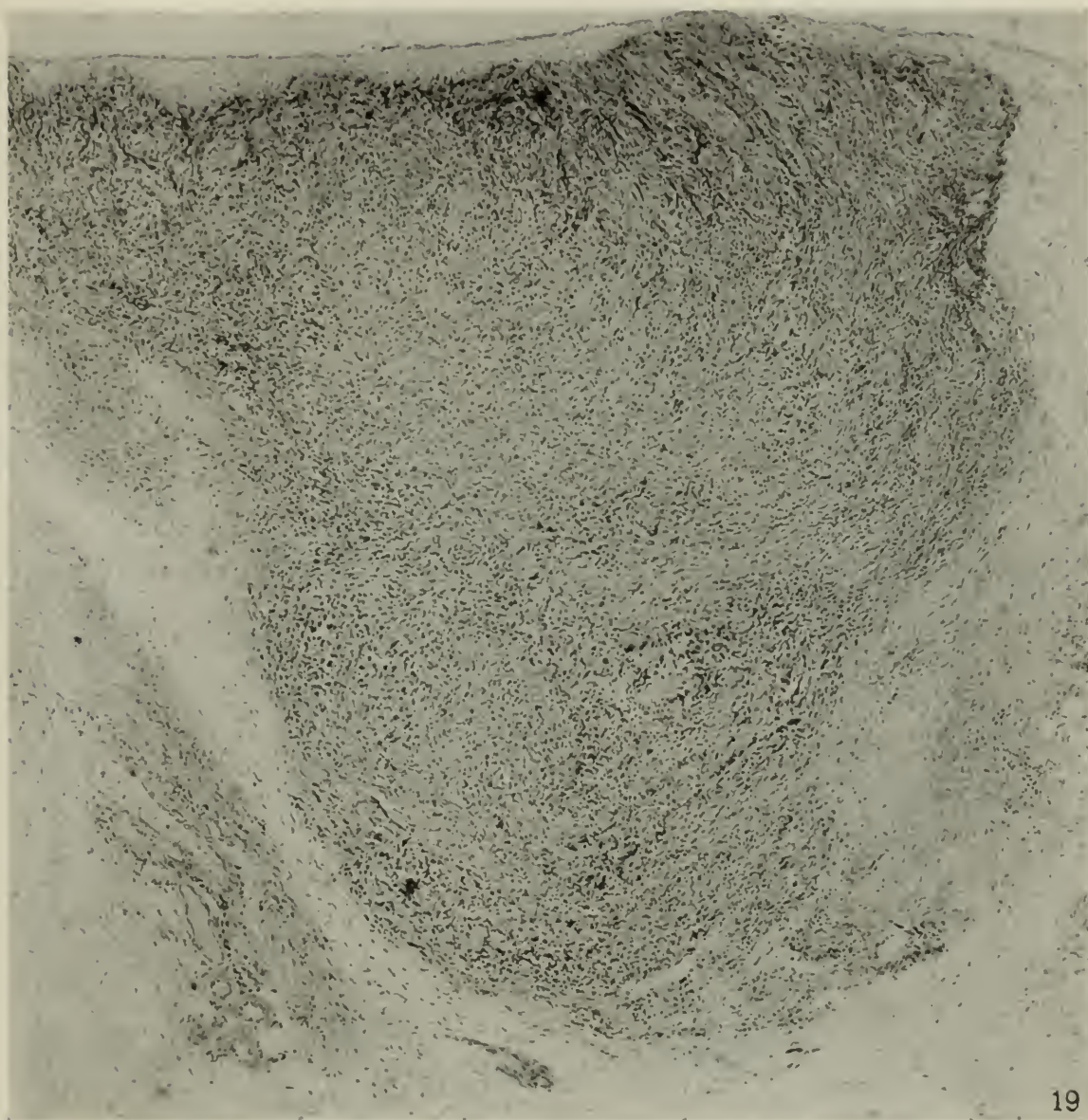
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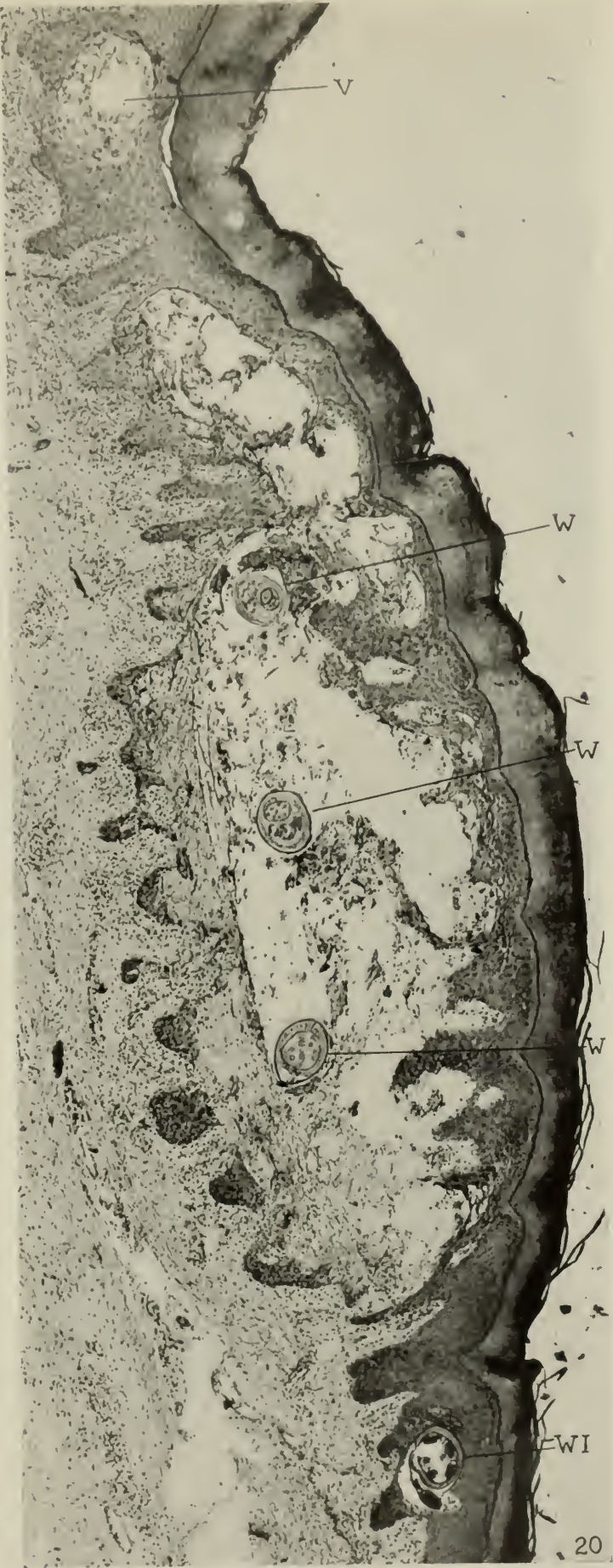


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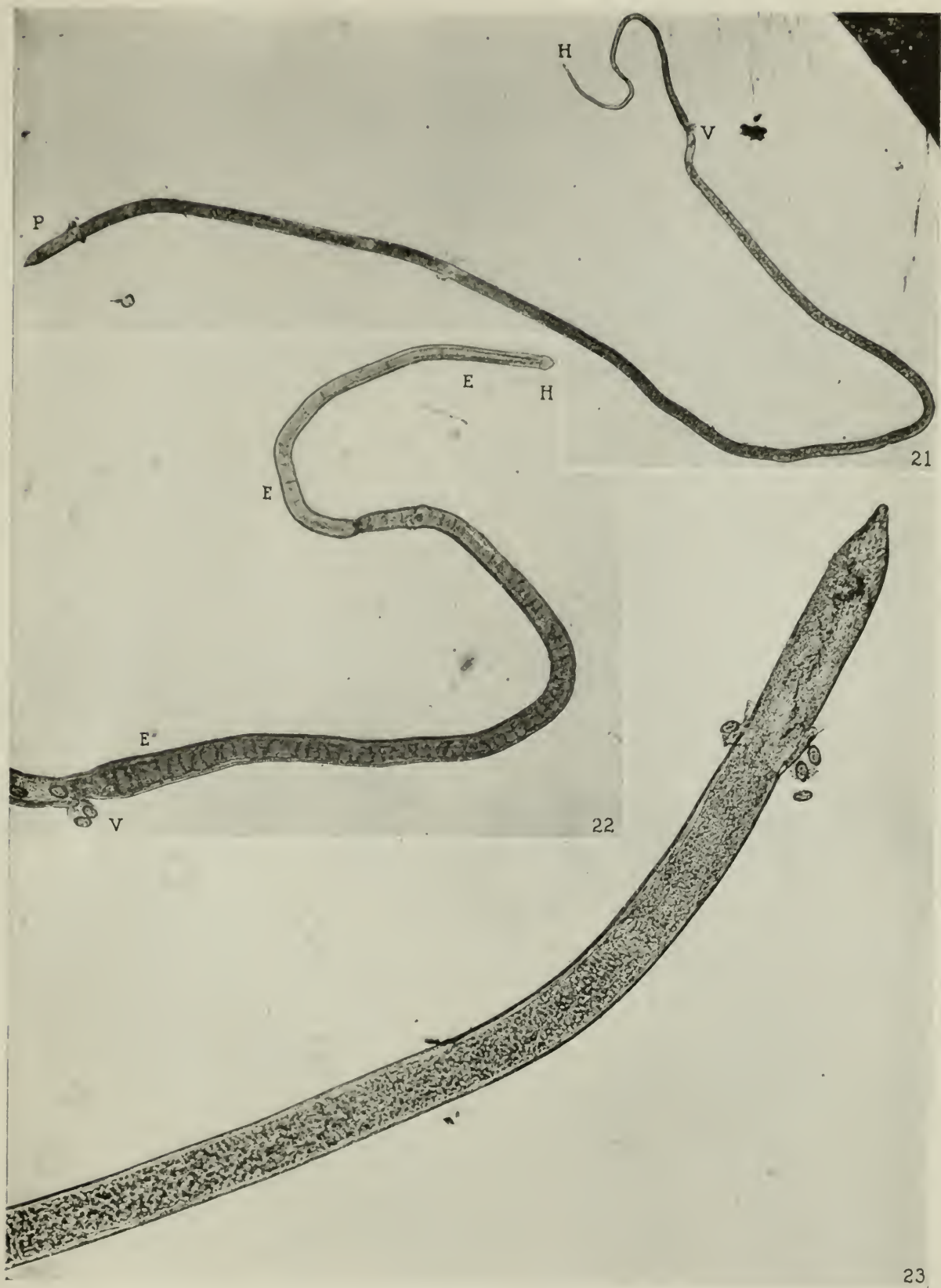


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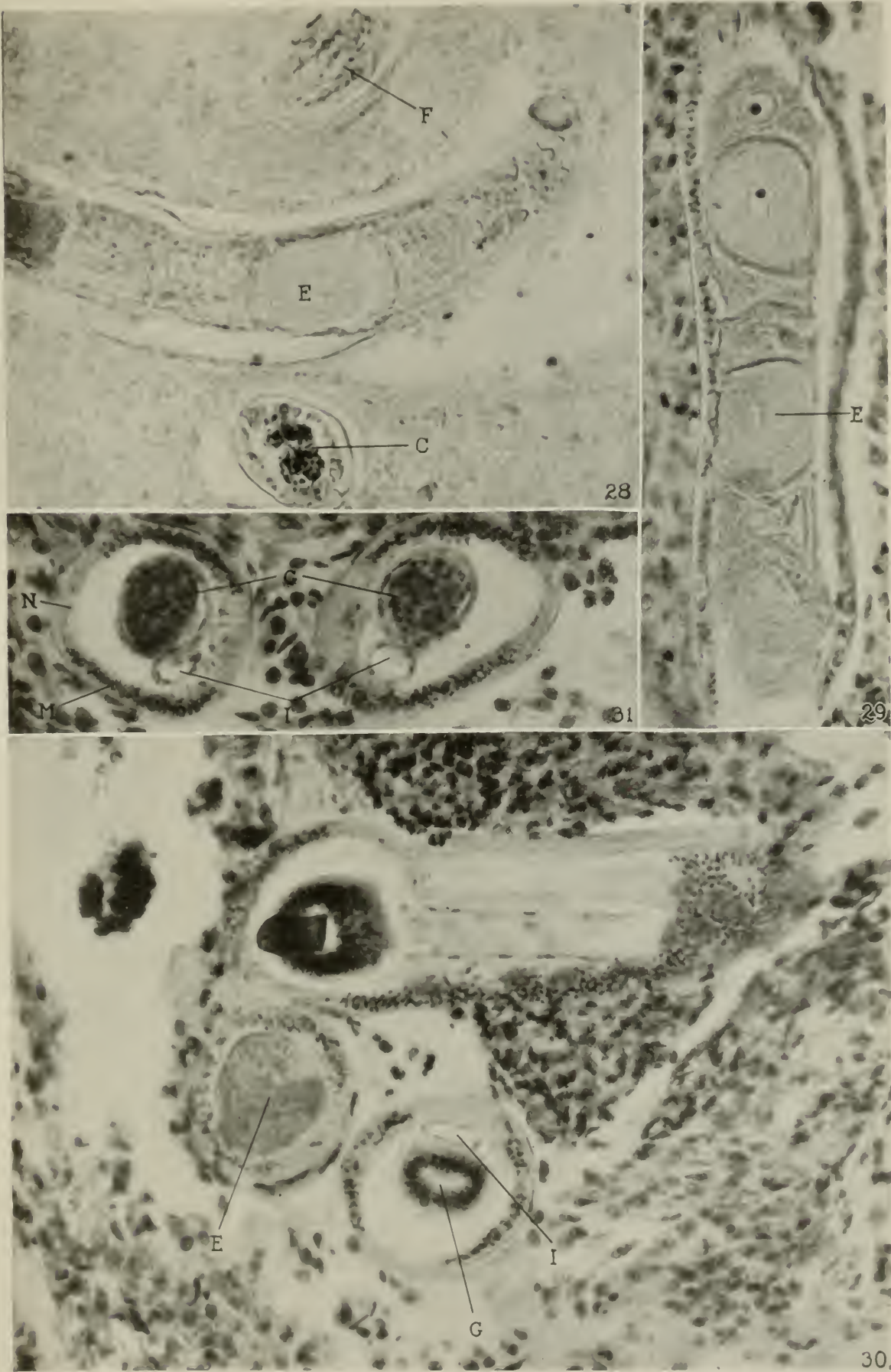


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(Swift, Boots, and Miller: Cutaneous nematode infection in monkeys.)

VIRULENCE AND MUTATION OF THE BACILLUS OF RABBIT SEPTICEMIA.

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INTRODUCTION.

Types D and G, bacillus of rabbit septicemia, have been shown to possess greatly differing degrees of virulence (1). Type G, of low virulence, has been demonstrated to arise as a mutant from the primordial highly virulent Type D (2). It is the purpose of this paper to inquire into the fixity of the character of virulence of the two types and to determine the relation that mutation bears to this function of invasibility.

Method.

The method of virulence determination employed in the following experiments is identical with that described in a previous paper (1). Briefly, it consists in the injection of varying amounts of 6 hour 10 per cent rabbit serum broth cultures, intrapleurally, into young rabbits of about 600 gm. weight. The dilutions of the test culture are carried out in plain broth, pH 7.4, and are increased by 10's. The dilutions are designated as follows: 0.0001 cc. of the original culture is written 10^{-4} cc., which of course is an abbreviation for 1×10^{-4} . The injections were invariably made immediately after dilution. The highest dilution was injected first, this procedure permitting the same syringe to be used for all injections. The final volume of each inoculation was in all cases 0.5 cc.

EXPERIMENTAL.

The persistence of the character of virulence of Type D is demonstrated by the following observations.

Experiment 1. Persistence of the Virulence of Strain R19, Type D.—Strain R19, Type D, was isolated on October 20, 1920, from the heart's blood of a rabbit dead of bronchopneumonia. The necropsy showed the typical bronchopneumonia and fibrinopurulent pleuritis and pericarditis, caused by the rabbit septicemia bacillus. This culture was placed in 10 per cent rabbit serum agar and transplanted at intervals of 7 days—incubation at 37°C. for 8 hours, the remainder of the time in the ice box. Virulence was tested on November 5, 10, and 14, 1920, and February 2, 1921; that is, 16, 21, 25, and 105 days after isolation. The results are summarized in Table I.

TABLE I.
Persistence of the Virulence of Strain R19, Type D.

Strain.	Length of time after isolation.	Weight of rabbit.	Age of serum broth culture injected.	Amount injected in- trapeurally.	Result.
	<i>days</i>	<i>gm.</i>	<i>hrs.</i>	<i>cc.</i>	
R19-D	16	550	6	10 ⁻⁵	Died in 15 hrs.*
		550		10 ⁻³	" " 13 "
		560		10 ⁻¹	" " 13 "
R19-D	21	600	6	10 ⁻⁶	" " 14 "
		650		10 ⁻⁵	" " 14 "
R19-D	25	525	6	10 ⁻⁹	Survived.
		515		10 ⁻⁸	Died in 12 hrs.
R19-D	105	650	6	10 ⁻⁸	" " 15 "
		650		10 ⁻⁷	" " 15 "
		650		10 ⁻⁶	" " 15 "
		650		10 ⁻⁵	" " 15 "

* The necropsy showed typical bronchopneumonia, fibrinopurulent pleuritis, and pericarditis.

Table I indicates that the virulence of this strain remains at a very high level over a comparatively long period of time. The high dilutions (10⁻⁸, 10⁻⁷) were plated in serum agar. 10⁻⁸ cc. yielded from three to eight colonies, showing the number of viable organisms in a 6 hour culture to vary from 300 to 800 million per cc. Strain R19-D was the most violently invasive of any of the cultures isolated. It is interesting to observe that the time of death of the test animals shows very little variation (12 to 15 hours). For the majority of virulent

Type D strains this is somewhat longer; *i.e.*, from 30 to 48 or 72 hours. Other Type D strains have been subjected to similar tests and, like Strain R19-D, exhibit a like constancy in maintenance of a high degree of virulence.

It was of interest to determine whether or not individual members of a single Type D culture were possessed of greatly varying degrees of virulence.

Experiment 2. Virulence of Six Pure-Line Strains, Type D, Fished from a Single Culture.—Six pure-line strains of Type D, R15, were fished by Barber pipette (3) from a 3 hour undiluted rabbit serum culture. These were seeded into undiluted rabbit serum and after several passages in this medium were transplanted to 10 per cent rabbit serum broth, incubated for 6 hours at 37°C., and tested for virulence by the usual technique. The results are summarized in Table II.

TABLE II.

Virulence of Six Pure-Line Strains, Type D, Fished from a Single Culture.

Strain.	Weight of rabbit.	Age of serum broth culture injected.	Amount injected intrapleurally.	Result.
	<i>gm.</i>	<i>hrs.</i>	<i>cc.</i>	
B-D ₁	600	6	10 ⁻⁶	Died in 36 hrs.*
	580		10 ⁻⁴	" " 36 "
B-D ₂	600	6	10 ⁻⁶	" " 36 "
	600		10 ⁻⁴	Survived.
B-D ₃	610	6	10 ⁻⁶	Died in 48 hrs.
	590		10 ⁻⁴	" " 47 "
B-D ₄	600	6	10 ⁻⁶	" " 36 "
	600		10 ⁻⁴	" " 36 "
B-D ₆	610	6	10 ⁻⁶	" " 36 "
	610		10 ⁻⁴	" " 48 "
B-D ₈	600	6	10 ⁻⁶	" " 36 "
	590		10 ⁻⁴	" " 36 "

* The necropsies were typical in all cases.

Table II indicates that there is very little variation in different individuals of a Type D culture. All of the six serum broth subcul-

tures from the pure-line strains were streaked on serum agar at the time of the virulence test and were found to contain Type D organisms only.

It is well known that various microorganisms attenuate quickly when they are isolated from the animal body and grow upon artificial media. This loss of virulence appears especially rapidly when the media employed are not enriched with blood, serum, or ascitic fluid. Wadsworth and Kirkbride (4) found that the virulence of pneumococci could be maintained at a high level for a long period when the culture was transplanted continuously at 8 hour intervals in plain broth, but that it quickly decreased when 24 hours were allowed to elapse between passages. Various observations having indicated that the virulence of Type D shows little variation, an experiment was planned to determine whether decidedly unfavorable conditions would depress this function. It has been shown in a previous communication that pure-line strains of Microbe D undergo mutation into Type G when allowed to stand without transplant for some days in plain broth. This process does not, however, go to completion under these conditions. A certain proportion of Type D remains as is evidenced by streaking such cultures on serum agar plates. It was proposed, therefore, to test the virulence of those Type D organisms which remain without change and at the same time to find out whether the newly mutated Type G colonies would yield cultures of characteristically low invasive power.

Experiment 3. Persistence of the Virulence of Type D in Broth Cultures Kept for 9½ Days at 37°C. without Transplant; Lack of Virulence of Type G Mutants Arising in This Culture.—0.05 cc. of an 18 hour rabbit serum culture, Strain A-D, R15, was seeded into a large test-tube containing 10 cc. of plain broth, pH 7.4. The tube was capped with tin-foil and placed in the incubator at 37°. The tube was removed from the incubator at 24 hour intervals and a loop of its contents streaked on 10 per cent rabbit serum agar plates by the method described in a preceding paper (2). Type G colonies began to appear on the plates of the 52 hour test, and at 228 hours (9½ days) Type G showed preponderance over Type D, the ratio between D and G being 40:60. Characteristic fluorescent Type D and non-fluorescent Type G colonies were now carefully fished to 10 per cent rabbit serum broth. Good growth had occurred in broth of the subcultures at 8 hours, the appearance of Type D being diffusely turbid, that of Type G very granular. Both tubes were now carefully shaken up, diluted to 10^{-7} cc. of the

original in plain broth, pH 7.4, and immediately injected intrapleurally into 700 gm. rabbits. The results of this experiment are recorded in Table III.

TABLE III.

Persistence of the Virulence of Type D in Broth Cultures Kept for 9½ Days at 37°C. without Transplant; Lack of Virulence of Type G Mutants Arising in This Culture.

Type.	Age of serum broth culture injected.	Weight of rabbit.	Amount injected intrapleurally.	Result.
	hrs.	gm.	cc.	
Type D; from plain broth culture; 228 hrs.; 37°C.	8	700	10 ⁻⁷	Died in 42 hrs.*
		710	10 ⁻⁶	" " 36 " *
		700	10 ⁻⁵	" " 36 " *
		690	10 ⁻⁴	" " 36 " *
		700	10 ⁻³	" " 42 " *
		720	10 ⁻²	" " 22 " *
		680	10 ⁻¹	" " 18 " *
Type G; from plain broth culture; 228 hrs.; 37°C.	8	700	10 ⁻⁴	Survived.
		710	10 ⁻³	"
		700	10 ⁻²	"
		710	10 ⁻¹	"
		700	5 × 10 ⁻¹	Died in 15 days. †

* Necropsy typical.

† Necropsy showed no evidence of infection; heart's blood sterile.

It will be observed from Table III that the subculture from the Type D colony was very virulent. Injected over a range of dilutions from 10⁻⁷ to 10⁻¹ cc., it proved fatal in every case. On the other hand, 0.1 cc. of the subculture from the Type G colony failed to produce an appreciable ill effect. The rabbit receiving 0.5 cc. succumbed in 15 days, but necropsy failed to reveal the characteristic bronchopneumonia and fibrinopurulent pleuritis and pericarditis, and the heart's blood culture proved sterile. This experiment is a clear demonstration that Microbe D is able to preserve its virulence under conditions generally considered to be unfavorable, while the mutant Type G forms that have arisen in the same culture show the lack of virulence that characterizes this type. It is apparent from the foregoing experiment that the virulence of a culture containing both Types D and G is proportional to the number of the D variety remaining in the culture.

If this be true, it is conceivable that the attenuation of a Type D culture in plain broth may be due to a $D \rightarrow G$ mutation and gradual increase in the number of Type G individuals. This augmentation might take place either by a constantly increasing tendency of the Type D forms to mutate, or by an actual overgrowth of Type D by the mutant G variety. If Type G should come finally to supplant the parent Type D completely, the formerly virulent culture should be almost completely attenuated. This idea was put to test in Experiment 4.

Experiment 4. Relation of the Virulence of a Plain Broth Culture to the Proportions of Types D and G Present.—Microbe D,R15, was transplanted daily for 25 days in plain broth, pH 7.4. From time to time the culture was streaked on 10 per cent serum agar plates. No Type G forms were observed. At the twenty-fifth passage the virulence of the culture was tested by the usual technique. At the thirtieth plain broth passage a few Type G colonies were detected on the serum agar plate. The culture was now transplanted every 48 hours. The relative number of Type G colonies increased rapidly. At the 51st passage Type G was found to preponderate greatly over Type D. The virulence of the whole culture was again tested at this point. The period of time between transplants was lengthened to 5 days. At the 56th passage serum agar plates revealed nothing but Type G. Microbe D had completely disappeared. The virulence was again tested. The results of the experiment are summarized in Table IV.

The results of Experiment 4, as seen in Table IV, can be summed up briefly as follows: At the twenty-fifth passage in plain broth no Type G colonies had arisen and the virulence was approximately equal to that of the original serum broth culture of Type D, 10^{-6} cc. proving fatal to 600 gm. rabbits. At the 51st passage, 2 months later, there was a large preponderance of Type G. The virulence had fallen to 10^{-4} cc. At the 56th passage, 5 days later, no Type D colonies could be demonstrated. Either complete $D \rightarrow G$ mutation had occurred, or the originally mutated Type G had completely outgrown Type D. 0.1 cc. of the culture failed to produce a fatal effect. The attenuation of this culture is to be referred to the gradual replacement of the primordial Type D by the mutant G form. It is possible to predict the virulence of a culture from the proportion of the two types present, as evidenced by the serum agar plate streak method. It is possible to procure subcultures of very high or very low virulence by selection of one type or the other, so long as any of Type D remain.

While the virulence of Type G, isolated from Type D cultures, is very low, large amounts of undiluted culture may occasionally cause fatal infections, especially in young rabbits. Adult rabbits of 1,200 to 1,800 gm. weight have not been observed to succumb, even when

TABLE IV.

Relation of the Virulence of a Plain Broth Culture to the Proportions of Types D and G Present.

No. of passages.	Interval between transplants.	Proportion of Types D and G.	Amount injected intrapleurally.*	Result.	Necropsy.
25	days 1	D only.	cc.		
			10^{-6}	Died in 36 hrs.	Typical. Type D in heart's blood.
			10^{-5}	" " 24 "	Typical. Type D in heart's blood.
			10^{-4}	Survived.	
51	2 (All passages from 25th to 51st at 2 day intervals.)	D + G; G greatly preponderates.	10^{-6}	Died in 31 hrs.	Typical. Type D in heart's blood.
			10^{-5}	Survived.	
			10^{-4}	"	
			10^{-3}	Died in 36 hrs.	Typical. Type D in heart's blood.
			10^{-2}	Survived.	
			10^{-1}	Died in 36 hrs.	Typical. Type D in heart's blood.
56	5 (All passages from 51st to 56th at 5 day intervals.)	G only.	10^{-5}	" " 36 "	Typical. Type D and few Type G in heart's blood.
			10^{-4}	Survived.	
			10^{-3}	"	
			10^{-2}	"	
			10^{-1}	"	

* All rabbits 600 to 650 gm. in weight. Cultures 6 hour, plain broth.

1.0 cc. of a whole culture of Type G has been injected. On the other hand, rabbits of 600 gm. weight at times are fatally infected by 0.5 cc., more often by 1.0 cc. of undiluted serum broth culture.

Experiment 5. Increase of the Virulence of Type G by Passage through Rabbits.—Microbe G was isolated from Strain R15-D and carried for thirty-eight passages in serum broth. It was then tested in the usual manner for virulence on rabbits of 600 gm. weight. Three rabbits of this series, injected with large doses, succumbed to typical infections indistinguishable from those caused by Type D. Pure Type G cultures were isolated in all cases. One of these was carried for five passages in serum broth and then retested for virulence. A strain of Type G, isolated from rabbits succumbing to the second virulence test, was carried in serum broth for fifteen additional passages, daily transplant, and again subjected to virulence test. The results are summarized in Table V.

The results summarized in Table V indicate that the virulence of Microbe G can be raised by passage. While the virulence undoubtedly increases, the other characteristics differentiating Type G from Type D persist and even intensify. This is especially true of the granular growth character in fluid medium. This becomes very intense upon animal passage, the organisms of serum broth cultures clumping into solid masses that fail to break up even after prolonged shaking of the culture tube. The acid agglutination zone also broadens considerably. Thus in the case of one of these intensely granular strains, a four times washed suspension of Microbe G agglutinated completely in all the tubes of an acetic acid-sodium acetate buffer series, from pH 5.6 to 3.0.

The experiment demonstrates that granular growth character and lack of virulence do not necessarily always occur concomitantly. It had been supposed that there might be some causal connection between the instability of the organisms and their lack of virulence, but this idea would seem to be ruled out by Experiment 5.

It will be observed that in the case of the last animal of the series in Table V some Type D colonies were observed on the serum plates streaked from the pleural fluid at necropsy. Do these represent reversion of Type G to the primordial D form? It is impossible to say whether or not this is the case. This is true because rabbits in many cases harbor Type D normally. The Type D discovered at necropsy might, therefore, simply represent individuals which have invaded the pleural cavity when the animal is already nearly dead from a true Type G infection. It is this tendency of normal rabbits to carry Microbe D that makes it unfeasible to attempt the reversion of Type G to the parent D variety. It is certainly true that Type G

TABLE V.

Increase of the Virulence of Type G by Passage through Rabbits.

Strain.	No. of passages.	Weight of rabbit.	Amount injected intrapleurally.	Result.	Necropsy.
		gm.	cc.		
R15-G	0	600	0.1	Survived.	No necropsy. Typical fibrinopurulent pleuritis, pericarditis, and bronchopneumonia. Pure culture Type G recovered from heart's blood.
		610	0.5	Died in 24 hrs.	
		600	1.0	Survived.	
		600	2.0	Died in 32 hrs.	
		600	3.0	" " 5 days.	
R15-G	1	620	10^{-3}	" " 16 hrs.	Congested lungs; increase in pleural fluid. Type G found in heart's blood at necropsy.
		610	10^{-2}	" " 16 "	Congested lungs; increase in pleural fluid. Type G found in heart's blood at necropsy.
		610	10^{-1}	" " 19 "	Beginning bronchopneumonia and pleuritis. Type G found in heart's blood at necropsy.
		600	5×10^{-1}	" " 16 "	Congested lungs; increase in pleural fluid. Type G found in heart's blood at necropsy.
R15-G	2	700	10^{-6}	Survived.	Lungs congested; pleural fluid increased. Heart's blood pure Type G. Lungs congested. Type G + few Type D found in heart's blood.
		750	10^{-4}	Died in 24 hrs.	
		725	10^{-2}	" " 36 "	
		700	10^{-1}	" " 15 "	

is by itself able to cause fatal infections when injected in overwhelming dose. This was the case in all of the other animals whose necropsies are recorded in the table.

What is the cause of the loss of the property of invasibility in the case of Type G? It was considered possible that Types D and G

might differ in degree of phagocytability or in their aggressive action against white blood cells. This idea was put to test in Experiment 6.

Experiment 6. Intrapleural Reaction to the Injection of Large Amounts of Types D and G.—9 hour serum broth cultures of Microbes D and G were carefully shaken up. 1 cc. of each of these was added to 1.5 cc. of plain broth. The suspensions of Type D and of Type G (total volume 2.5 cc.) respectively were injected intrapleurally into each of two rabbits of 1,600 gm. weight. Pleural fluid was aspirated after 1, 2, and 4 hours, and smears were made of the withdrawn fluid and stained with Löffler's methylene blue. The results of this experiment are given in Table VI.

TABLE VI.

Intrapleural Reaction to the Injection of Large Amounts of Types D and G.

Interval before examination.	Microbe D.	Microbe G.
	9 hr. serum broth culture intrapleurally in 1,700 gm. rabbit.	9 hr. serum broth culture intrapleurally in 1,700 gm. rabbit.
<i>hrs.</i>		
1	No fluid obtainable.	No fluid obtainable.
2	Large amount of fluid; myriads of typical bipolar organisms; no slight phagocytes.	Small amount of fluid; no phago- cytes; a few clumps of typical bipolar organisms.
4	Myriads of typical organisms; few phagocytes, many badly damaged; slight phagocytosis.	Many phagocytes with ingested or- ganisms; no free organisms ob- served.
16	Dead; myriads of organisms; some phagocytosis.	No free organisms; few phagocytes. Survived.

It would appear from the experiment just described that Type D possesses a strong antagonistic effect upon the phagocytes, which allows the microbes to gain their primary foothold in the pleural cavity and undergo intense multiplication. The mutant Type G organisms appear to have lost this aggressive activity and are rapidly clumped and ingested by the white blood cells, which appear at the injection site within 4 hours after inoculation. These facts would seem to furnish a clue for the study of possible aggressive substances possessed by Microbe D.

It has just been shown that Type G is rapidly disposed of when injected intrapleurally. It was interesting to observe that a culture of Type G which produced no perceptible effect when injected into

rabbits intrapleurally in dose of 1.0 cc., gave rise to abscesses when injected subcutaneously in 0.1 cc. amount. These abscesses were sharply circumscribed, purulent, and teeming with typical G microbes. In no case did the organisms generalize and death result. Type G could be recovered from them for several weeks. This demonstrates a vicariously greater susceptibility to infection depending upon the route of injection. It is well known that phagocytes are more rapidly mobilized on serous surfaces than subcutaneously, and in the light of Experiment 6, the phenomenon of greater infectivity of Type G by subcutaneous route would seem to be due to greater time necessary to bring the phagocytes into play in this region.

DISCUSSION.

The importance of the foregoing observations and experiments is threefold.

1. Various authors have observed the appearance of granular growing varieties in cultures of various microorganisms. Such types have been reported by Arkwright (5) and by Zoeller (6) for *Bacillus dysenteriae* Shiga. They have been noted in the case of the hog-cholera bacillus by Krumwiede and Provost¹ and by Krumwiede and Valentine for pneumococcus.² This phenomenon is not therefore confined to cultures of the rabbit septicemia bacillus and may be found to be of general occurrence. It would be of interest to observe whether lack of virulence is associated with granular growth in these instances.

2. The occurrence, side by side, of the two varieties, the parent Type D, highly virulent, and its mutant Type G, of low invasibility, furnishes an excellent opportunity for the study of the mechanism of virulence. It is possible to investigate the products of secretion and of cell destruction of the virulent Type D, with a view to discovering whether these substances, when added to Type G, might increase its power of invasion. In brief, Type G may be used as a reagent with which to attempt the discovery of the factors responsible for the high invasive power of Type D.

¹ Personal communication.

² Personal communication.

3. It is important to find out whether the G forms appear only in strains which have been artificially cultured for some time outside the animal body, or whether they may occur naturally in the animal as well as in the test-tube. This question would seem to have a direct bearing upon the problem of epidemiology. It is at present under investigation and certain results have already been obtained, which will be reported in the near future.

SUMMARY AND CONCLUSIONS.

Type D, bacillus of rabbit septicemia, exhibits marked fixity of the character of virulence. This is true for cultures that are regularly transplanted on serum agar or in broth. It is also to be observed when organisms of this variety are subjected to unfavorable conditions; *e.g.*, remaining for $9\frac{1}{2}$ days without transplant in plain broth. Under such conditions no decrease in virulence was observed. G forms which arose in the same culture during this time exhibited characteristic lack of invasibility.

Different individuals of a given culture of Type D do not vary to a noticeable extent in virulence. This was ascertained by test of virulence of cultures arising from six individuals fished from Type D cultures by Barber pipette.

A Type D culture subjected to passage in plain broth undergoes $D \rightarrow G$ mutation. Type D and G individuals can be demonstrated to be present in the same culture. The virulence of such a mixed culture is proportional to the relative number of Microbes D and G present. This throws light on certain mechanisms of attenuation.

The virulence of Type G can be raised to a considerable titer by animal passage. Such organisms do not, however, lose their characteristic of granular growth. This last, on the other hand, appears to intensify by animal passage. The acid agglutination zone of Type G strains which have been passed through animals shows a marked broadening.

Microbe D owes its superior invasive power at least in part to its antiphagocytic activity, a property apparently not possessed by Microbe G.

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COLLODION SACS FOR AEROBIC AND ANAEROBIC BACTERIAL CULTIVATION.

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PLATES 53 AND 54.

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Among the various media for bacterial cultivation, one that has proved especially valuable for the isolation and maintenance of highly parasitic organisms is a combination of ascitic fluid or dilute blood serum and a fragment of fresh animal tissue. Such a medium was suggested by Theobald Smith¹ and has been extensively developed and used by Noguchi² in the cultivation of spirochetes, the globoid bodies of poliomyelitis, and other organisms whose isolation in pure culture he has reported from time to time. *Bacterium pneumosintes*,³ recovered by Olitsky in 1918 from the filtered nasopharyngeal secretions of patients with epidemic influenza, was isolated under strictly anaerobic conditions in a combination of sterile human ascitic fluid and fresh rabbit kidney tissue.

A certain disadvantage of the medium is found in the presence of protein precipitate derived from autolysis of the tissue fragment. Both macroscopically and in stained specimens this precipitate may obscure the view and either simulate or mask the presence of significant bodies. This disadvantage is apparent especially in the search for hitherto undiscovered microbes and in dealing with filter-passing organisms such as the globoid bodies of poliomyelitis or *Bacterium pneumosintes*, which are just within the limits of vision and are made out with difficulty in early generations. Furthermore, the antigenic protein precipitate makes tissue cultures unsuitable for the production

¹ Smith, T., *J. Boston Soc. Med. Sc.*, 1898-99, iii, 340.

² Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

³ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

of immune serum or for serological studies in general, because it gives rise to unspecific reactions and so complicates the results.

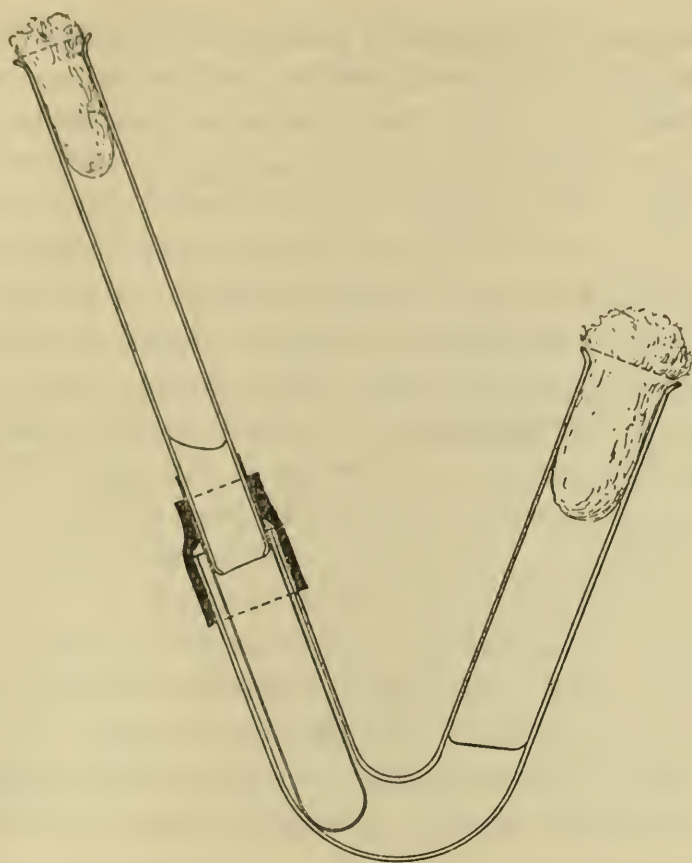
In the course of recent studies on *Bacterium pneumosintes*, it became desirable to cultivate the organism in a medium without antigenic properties and free from the confusing protein cloud. For this purpose it was proposed to confine the tissue medium in a collodion sac surrounded by distilled water or salt solution, with the prospect that the nutritive and growth-promoting substances of the medium would diffuse through the sac wall and support growth in the surrounding fluid. The design of the apparatus was limited by the necessity for oxygen exclusion and for adjustments of osmotic pressure, so that the collodion sacs prepared for intraperitoneal implantation⁴ were found to be unsuitable. It was also desirable that the dialysate cultures might be made in considerable numbers and be subject to frequent examination without disturbance of their anaerobic state. These conditions were met in the method to be described. The apparatus is adapted to various problems in bacterial growth and metabolism, and may therefore find a wider application than we originally intended. Two designs of apparatus have been developed, one containing about 10 cc. of culture medium for routine cultivation, the other holding 50 cc. for mass cultures. The principles involved are the same, but differences in preparation necessitate separate descriptions.

Collodion Sacs in V-Tubes.

For small amounts of culture media a V-shaped glass tube⁵ similar to the Smith fermentation tube, except that both ends are open, incloses the collodion sac and its surrounding fluid. The sac almost fills one limb of the V, reaching to the bend at the bottom, and is surrounded by the dialysate fluid, which rises a short distance in the other limb and is thus accessible from its open end. The mouth of the sac is shrunk onto a glass tube sealed into the V-tube by a rubber collar (Text-fig. 1). For anaerobic cultivation the medium in the sac and the dialysate fluid are layered with vaseline, or the V-tube may be placed in an anaerobic jar.

⁴ Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.

⁵ Kimble Glass Co., Vineland, N. J., or Corning Glass Works (Pyrex), Corning, N. Y.



TEXT-FIG. 1. Cross-section of a collodion sac in its V-tube ready for sterilization.

Preparation of the Collodion Sacs.

The preparation of a considerable number of identical collodion sacs in the simplest and most mechanical manner involves the assembly of some accessory apparatus which adds greatly to the ease and rapidity of the method.

The molds in which the sacs are formed are clusters of five short round bottom test-tubes 10 by 1.4 cm. inside, thrust to the lip through holes in a large (No. 13) rubber stopper (Fig. 1). This stopper fits the mouths of three cylindrical museum jars, 5.5 by 15 cm., provided with glass tops, gaskets, and clamps.⁶ One jar contains a 10 per cent solution of gelatin, preserved with 0.3 per cent tricresol; the second contains a thick collodion solution; the third is partly filled with alcohol.

⁶ Whitall Tatum Co., New York, N. Y., Fig. 2600, No. 2.

For the purpose of drying the gelatin coating and the collodion membrane with which the molds are lined, we have set up an apparatus to blow air into the molds. Short stubs of glass tubing are thrust through a No. 13 rubber stopper in a pentagonal pattern corresponding to the molds. This stopper is set in a small glass funnel for the delivery of air, and the funnel and stopper are supported in a ring clamp and held in place with adhesive plaster. A second ring clamp supports the rubber stopper containing the cluster of molds above the glass tips, so that a jet of air may be directed into each.

The molds are first lined with a thin coat of gelatin, which prevents the collodion membrane from adhering to the glass. The ease with which the completed sacs are withdrawn from the molds justifies this preliminary procedure. To line the molds, which must be clean and smooth, the gelatin solution is melted in a bath of warm water, the cluster of molds is fitted to the mouth of the jar, and the jar is momentarily inverted. The excess gelatin drains back into the jar and any bubbles in the gelatin break while the film is cooling. This thin coat of gelatin dries in a few minutes over the air jets described above. A number of mold clusters are prepared before the collodion sacs are made.

The collodion solution should be so thick and viscous that a membrane of sufficient thickness and strength will adhere to the mold even after 3 to 5 minutes drainage. Such a thick collodion is prepared most easily by evaporation of a thinner solution in a partial vacuum with gentle heat, as described in a former paper.⁴

To form the collodion membranes, a cluster of molds is fitted to the museum jar containing collodion, the jar is inverted momentarily, and the excess liquid is allowed to drain back into the jar until drops no longer form and fall. It may be necessary to hold the stopper tightly in place during the inversion, as evaporation of the solvents produces a slight pressure in the jar. A long drainage produces walls of more uniform thickness and allows even minute bubbles in the membranes to drain out or to break.

The collodion-coated molds are then removed from the jar, which is quickly covered again, and are held over the air jets for a few seconds until the ether vapor is washed out and the collodion sets. As was pointed out in the former paper,⁴ the membranes must not be allowed

to dry completely if their permeability is to be retained after heat sterilization. As soon as the collodion sets, the molds are filled with alcohol by inversion of the third museum jar. Here they are allowed to soak for about 10 minutes, during which time the jar may be reversed two or three times to bring fresh alcohol into the tubes.

The molds are then rinsed out several times in cold tap water and immersed for a few minutes while the water penetrates the membranes and softens the gelatin beneath. During this immersion in cold water the lips of the tubes are scraped with a knife to cut the sacs free from the layer of collodion on the rubber stopper and each sac is fitted with the glass tube which is to support it. These glass tubes, 16 by 1.3 cm. outside, with an elastic band snapped on 2 cm. from one end, are of such a size in relation to the molds that they will just slip down into the sacs until checked by the elastic band.

When the gelatin lining under the collodion has softened, the molds with the glass tubes surmounting them are lowered for a minute or two into boiling water in a beaker or water bath. A wire hook (Fig. 1) which engages a small staple centrally placed among the lips of the molds is convenient for this purpose. The hot water shrinks the sacs down tightly upon the glass tubes, melts the gelatin, and so frees the sacs from the molds, so that when the tubes are withdrawn, the sacs come with them. The elastic bands are slipped down over the upper edge of the sacs, holding them in place securely.

The sacs are then rinsed in warm water to remove any traces of gelatin. When empty, they may be tested for leaks by inflation under water. They are now ready for insertion into the V-tubes. These tubes, of 1.5 cm. internal diameter, have limbs 6 and 12 cm. long. A wet collar of pure gum rubber tubing is slipped onto the shorter limb so that it does not obstruct the open end, into which the sac is thrust until its glass-protected neck is in the opening and its closed end has reached the curve at the bottom of the V-tube. The collar is then slipped up to hug the neck of the sac, which is allowed to project a millimeter or two above the collar. Thus the sac is held in place and the inner and outer chambers of the apparatus are sealed (Text-fig. 1). If rubber tubing of the proper size is not available, the collar may be made of a piece of thin rubber dam, 15 by 5 cm., wrapped tightly around the juncture of the V-tube and the sac, and secured with rubber bands.

The sac is then filled and surrounded with distilled water or saline solution, the ends of the tubes are plugged with cotton, and the completed apparatus is sterilized in the autoclave. Finally, the junction between rubber collar, sac, and tube is painted with hot paraffin wax. The V-tubes are carried in test-tube racks cut with slots instead of round holes.

Collodion Sacs in Flasks.

For mass cultures, collodion sacs of about 50 cc. capacity have been prepared. The molds are 50 cc. Erlenmeyer flasks, lined with a gelatin film. The melted gelatin is poured from one flask to another until all are coated. They are drained mouth downward in a rack (Fig. 2), until the gelatin cools and sets. The film is then dried over a jet of air. Collodion is kept in 120 cc. wide mouth rubber-stoppered bottles, into which the filled flasks are allowed to drain until all bubbles have broken and the excess collodion has dripped away. The subsequent treatment is the same as with the smaller sacs. Rather larger glass tubes, 16 by 1.6 cm., are required to support the sacs, and the elastic bands must be stretched tightly to prevent leaks between membrane and tube. The band is put on by collapsing the sac in a 50 cc. centrifuge tube or wide test-tube, around which the rubber band has been tightly doubled and wound. The neck of the sac, with the glass tube in position, projects from the centrifuge tube. The rubber band is then snapped off into place.

As containers, we have had glass tubes 12 by 1.5 cm. fused into 100 cc. Pyrex Erlenmeyer flasks near the bottom, thus forming a spout which gives access to the space surrounding the sac. The sac is collapsed, inserted into the flask, and sealed in place by an elastic collar of tubing or rubber dam. The necks of sacs made in ordinary 50 cc. flasks are not long enough to extend above this collar, hence the necessity for a tight rubber band around the neck. We have had no leakages which could be traced to this source. As with the smaller sacs, the apparatus is partly filled with distilled water or salt solution, plugged with cotton, and autoclaved. The juncture of collar and tube is then paraffined.

Additional Notes on the Preparation and Use of the Sacs.

In order not to prolong the foregoing descriptions, a number of important steps in the preparation of the sacs were not emphasized or discussed. Most important among these steps are those that determine permeability. This subject was treated at some length in the former paper on collodion sacs, and will not be reiterated here. But attention may be called to several facts which have more recently been noted.

Composition of the Collodion.—We have used a commercial collodion,⁷ evaporated in a partial vacuum until thick enough to give a strong membrane with a single coating of the molds. A sufficiently high degree of permeability is conferred upon the membranes by immersion in 95 per cent alcohol, as recommended by Brown.⁸ Eggerth,⁹ who credits Malfitano with a prior observation, has found that the relative amounts of alcohol and ether in the collodion determine permeability, and has described a series of membranes of graded permeability produced by varying their alcoholic content. Eggerth also found that the addition of 10 to 30 per cent of lactic acid to the collodion greatly increases the permeability of the resulting membranes. These membranes are transparent, tough, and elastic, and of such a high degree of permeability that they readily pass the proteins of serum and ascitic fluid. This is a drawback to their use with a tissue medium when a bacterial growth free from foreign protein is desired. By varying the alcoholic content of the collodion, or by the addition of lactic acid, or by immersion of the undried membrane in alcohol solutions of varied concentrations, a wide range of permeabilities may be obtained.

Eggerth observed that alcohol-rich collodions are made more viscous by the addition of water, and that the presence of even traces of moisture causes them slowly to set. We have had the same experience with thick collodion solutions. Only by keeping the collodion dry can a thick solution be maintained in a fluid state.

⁷ Squibb's Contractile Collodion, U. S. P.

⁸ Brown, W., *Biochem. J.*, 1915, ix, 591.

⁹ Eggerth, A. H., *J. Biol. Chem.*, 1921, xlviii, 203.

Sterilization by Heat.—The shrinkage and the loss in permeability of collodion membranes when subjected to sterilization in the autoclave were discussed in the former paper. This shrinkage indicates a change in plasticity and cohesion under the influence of heat. Membranes immersed in alcohol are highly plastic and will stretch considerably under pressure without breaking. Thus a sac with a capacity of 45 cc. was stretched to a capacity of 77 cc. by an internal pressure of 12 cm. of 95 per cent alcohol, when its further expansion was checked by the container. On replacement of the alcohol by water, collodion membranes become more rigid, so that water pressures up to the breaking point may be withstood without change in size. If the membranes are heated, however, their plasticity is increased, and the shape that a sac assumes is the result of a balance between the forces of cohesion and the pressure applied to the sac wall. At atmospheric pressure the membrane shrinks progressively as the temperature is raised, but this tendency may be counteracted by the application of pressure within the sac. A slight pressure prevents shrinkage; a greater pressure expands the sac just as it does a sac immersed in alcohol at room temperature. The following experiment illustrates this fact.

Experiment 1. Effect of Pressure on Heated Collodion Membranes. Sac A, Unheated Control.—Capacity 54.8 cc. Immersed in water at room temperature under an internal pressure of 30 cm. of water. After 15 minutes at this pressure, the capacity of the sac was still 54.8 cc. showing that the elastic limit of the membrane had not been exceeded.

Sac B, Heated at Atmospheric Pressure.—Capacity 59 cc. Immersed in a water bath at 20°C. Temperature raised to boiling over a period of about 9 minutes. Boiled 5 minutes. Capacity after heating 38.5 cc.

Sac C, Heated under an Internal Pressure of 25 Cm. of Water.—Immersed in a water bath at 20°C. (with Sac B). Temperature raised to boiling over a period of about 9 minutes. Boiled 5 minutes. Capacity after heating 75.5 cc.

The appearance of these sacs after the experiment is shown in Fig. 3. The relative plasticity of Sacs B and C during the heat treatment is further illustrated by the pear shape they have assumed. Sac A retains the shape of the mold in which it was made.

When a membrane has once been heated, it becomes permanently less plastic and subsequent reheating does not alter its shape and size to a similar extent.

Experiment 2. Effect of Pressure on Reheated Collodion Membranes.—Sac A had a capacity before heating of 52.7 cc. This sac was heated at atmospheric pressure and boiled in a water bath for 9 minutes. After heating, its capacity was 38.7 cc. The sac was again heated under an internal pressure of 32 cm. of water, and boiled for 5 minutes. The capacity of the sac was then 44 cc.

Sac B, capacity 53.5 cc., was boiled under a pressure of 32 cm. of water. The capacity of the sac increased to 68.5 cc. Then the sac was boiled for 5 minutes at atmospheric pressure. Its capacity was reduced to 64.5 cc.

If the shrinkage which occurs in the autoclave is undesirable, it may readily be prevented by sterilization under a slight internal pressure produced by filling the glass neck of the sac with water to a predetermined level, or by a preliminary immersion of the sac in boiling water under a similar water pressure. As tested by the rate of filtration of water under 25 cm. pressure, however, very little difference in permeability results from the prevention of shrinkage, or the expansion of the sac during sterilization by heat. Since it has been possible to prepare sacs which are too permeable for our purpose, the loss in permeability which an undried sac undergoes on sterilization has not been a drawback. Excessive shrinkage due to high temperature is to be avoided, however. We autoclave our sacs for 1 hour at 108°C. (5 pounds steam pressure).

Mode of Use of the Sacs.—The method of filling the sacs and the various uses to which they may be put hardly need a detailed description. Various applications will suggest themselves to meet individual requirements. The apparatus is convenient for either aerobic or anaerobic cultivation. For aerobes the space around the sac should not be entirely filled with fluid. For anaerobes, all bubbles should be tipped out of this space, and both limbs must be sealed with vaseline, or the cultures placed in an anaerobic jar. The tubes or flasks may be inoculated immediately, but we have found it advisable to incubate them first for 24 to 48 hours, to prove their sterility and to permit dialysis of nutrient materials. During this period anaerobic conditions will develop under a vaseline seal. It may be pointed out in this connection that while a seal of vaseline excludes oxygen, it also prevents the free escape of carbon dioxide from the medium. Retention of carbon dioxide may lead to a more rapid acidification of the medium than would otherwise occur.

Hydrogen Ion Concentration of the Dialysate.—In general, the dialysate acquires the hydrogen ion concentration of the medium within the sac. In a well buffered colloidal medium, however, the diffusion of substances which change the hydrogen ion concentration is relatively slow, and consequently the acid produced in the vicinity of a tissue fragment may not be neutralized by a supernatant column of alkaline medium, and an undue proportion of it may diffuse through the sac wall and acidify the dialysate. We have occasionally found it desirable, therefore, to buffer the dialysate or to bring it to a faint alkalinity with sodium hydroxide before inoculation. Phosphate mixtures in the absence of protein appear to be toxic to some bacteria. Peptone broth, when its use is not contraindicated, is an effective buffer and has been used both as a diluent for serum in the medium, and as a dialysate fluid. The addition of dextrose, 1 to 2 per cent, hastens the establishment of anaerobic conditions, and of course increases the nutritive value of the medium for organisms which can utilize it.

Osmotic Pressure.—The osmotic pressure developed in the system depends upon the permeability of the sac in relation to the composition of the culture medium. When a medium containing substances in solution that will not pass through the sac is dialyzed, osmotic pressure adjustments are made automatically by imbibition of water and a rise in the level of the medium within the sac. Thus in Fig. 4 the difference in the levels of medium and dialysate represents the osmotic pressure of a mixture of horse serum and dextrose broth dialyzed against distilled water. Imbibition of water may be prevented by filling the sac and its glass neck approximately to the osmotic pressure level, as found by experience with the given medium. The neck of the sac may be filled beyond the pressure level. Then a filtration of fluid from the sac into the surrounding liquid results that may aid the diffusion of nutritive substances. When collodion containing 10 per cent of lactic acid is used, and the sacs are treated with 95 per cent alcohol (also containing lactic acid), their permeability is such that serum proteins pass through freely, and no difference in levels is established (Fig. 5). Sometimes changes in level break a vaseline seal which is to be restored by gentle heating.

Symbiosis.—Eggerth⁹ found that *Bacillus influenzae* Pfeiffer could be grown in plain broth in a sac surrounded by a living culture of staph-

yllococcus, streptococcus, or pneumococcus. We have had a similar experience with *Bacterium pneumosintes*. *Bacillus subtilis* was grown in dextrose broth within collodion sacs. After 24 hours the growth was checked and anaerobic conditions were established by the addition of a vaseline seal. *Bacterium pneumosintes* then grew luxuriantly in dextrose broth surrounding the sacs, although it failed to grow in the same medium in control tubes without *Bacillus subtilis*.

It appears, therefore, that the growth-promoting substances, presumably catalytic or enzymatic in nature, which are necessary to the multiplication of microorganisms may diffuse through collodion membranes of high permeability.

Primary Isolation of Highly Parasitic Organisms.—What little experience we have had indicates that the Smith-Noguchi ascitic fluid-tissue culture is a more favorable medium for the primary cultivation of highly parasitic organisms than is the dialysate of such a medium obtained with collodion sacs. When growth on an artificial medium is once established, however, subplants of *Treponema pallidum*, *Treponema microdentium*, and *Bacterium pneumosintes* have grown readily in the dialysate fluid. The absence of precipitate from the medium permits the collection of a sediment for antigenic and serological purposes composed of the microbic bodies alone. In the case of *Bacterium pneumosintes*, this has made possible a study of its antigenic character.

SUMMARY.

A simple and convenient method is described for the preparation and use of collodion sacs for aerobic and anaerobic bacterial cultivation *in vitro*.

The sacs are suitable for the study of various problems in bacterial growth and metabolism. By their use with the Smith-Noguchi tissue medium, certain microorganisms may be grown in the absence of the confusing, antigenic protein precipitate characteristic of cultures containing fresh tissue, and thus the organisms are obtained in a suitable condition for serological study.

EXPLANATION OF PLATES.

PLATE 53.

FIG. 1. Solutions of collodion, gelatin, and alcohol in museum jars. Cluster of molds inserted in a rubber stopper which fits the jars. 50 cc. Erlenmeyer flask lined with a gelatin film. Hook for lowering molds into boiling water.

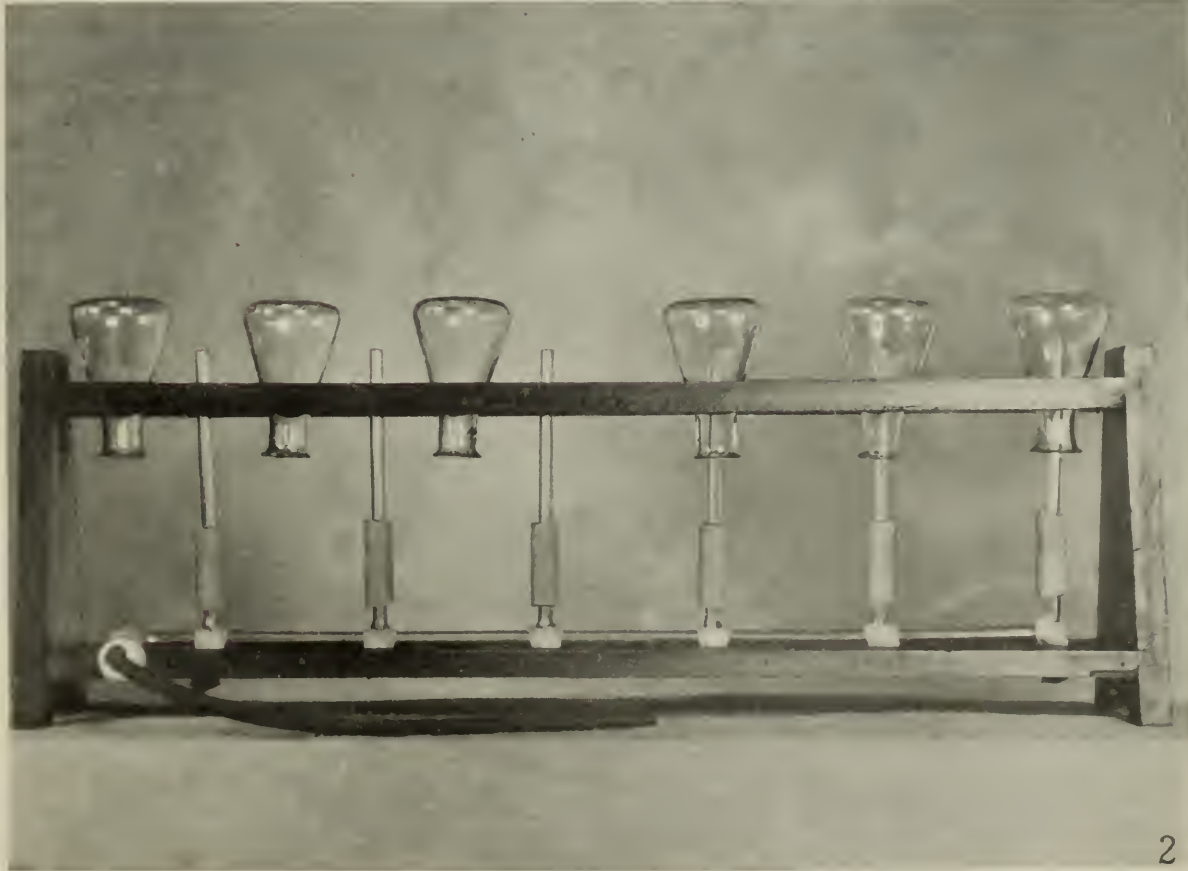
FIG. 2. Rack for draining and drying Erlenmeyer flasks lined with gelatin.

PLATE 54.

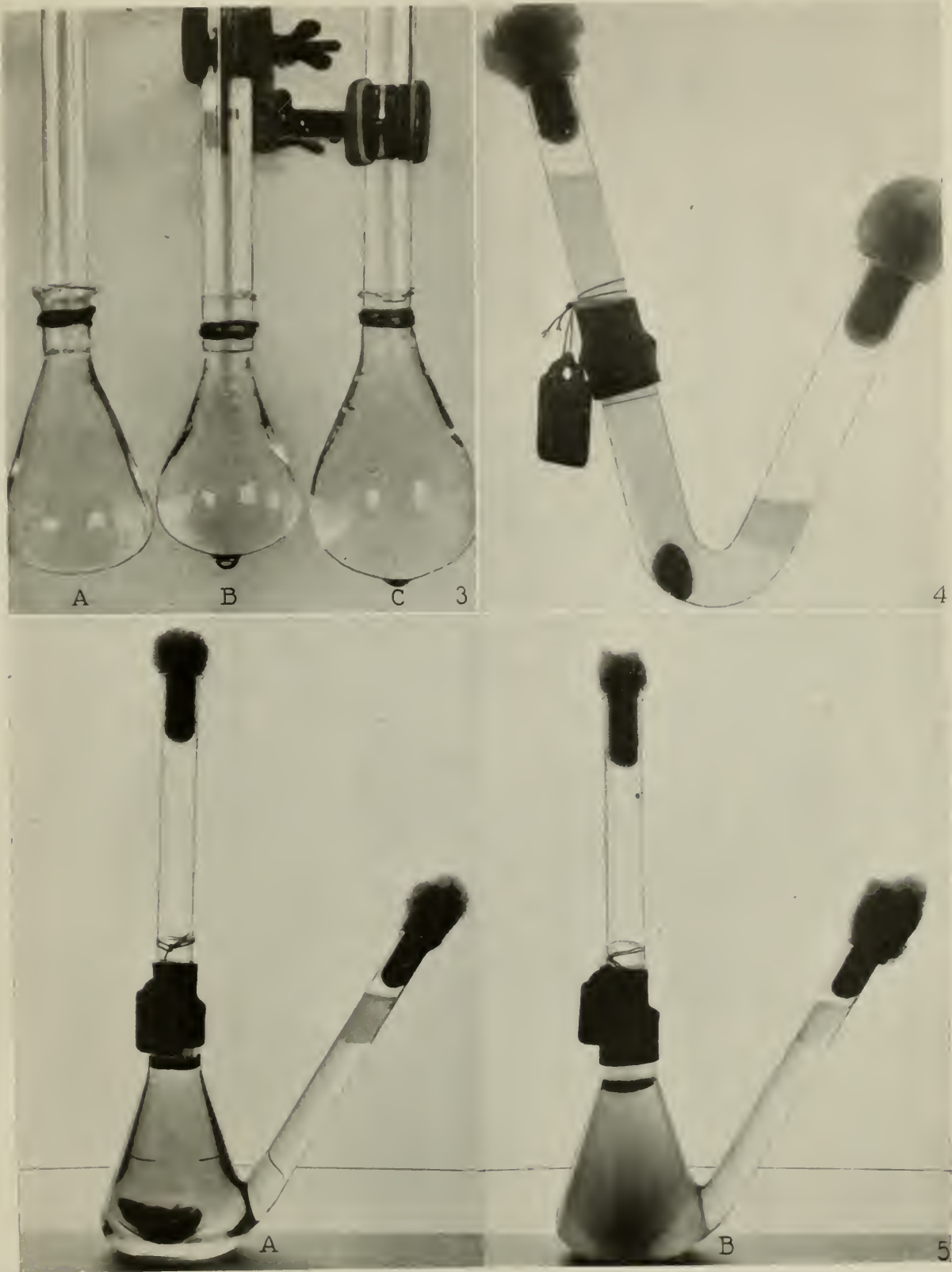
FIG. 3. Sacs described in Experiment 1. Sac A, unheated control; Sac B, heated under atmospheric pressure; Sac C, heated under an internal pressure of 25 cm. of water. Note the pear shape assumed by Sacs B and C.

FIG. 4. Sac system in a V-tube, containing a kidney tissue medium *versus* distilled water. Both liquids are layered with vaseline. The difference in levels indicates the osmotic pressure of a mixture of horse serum and dextrose broth.

FIG. 5. Flask A, a culture of *Treponema pallidum* in the dialysate of an ascitic fluid-dextrose broth-kidney tissue medium. The microorganisms do not cloud the fluid, but grow in feathery gray flocculi at the bottom of the flask. Flask B, a culture of *Bacterium pneumosintes* in the dialysate of a horse serum-dextrose broth-kidney tissue medium. As shown by the faint image of the string in the tube, the fluid is heavily clouded. These two sacs, made with lactic acid collodion, are so permeable that the level of the medium is not raised by osmotic pressure.



(Gates: Collodion sacs for bacterial cultivation.)



(Gates: Collodion sacs for bacterial cultivation.)

HEAT AND GROWTH-INHIBITING ACTION OF SERUM.

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INTRODUCTION.

It is known that plasma or serum obtained from an adult animal restrains the growth of a pure culture of homologous fibroblasts.¹ This inhibiting power of plasma or serum is not apparent in very young animals, but manifests itself a few weeks after birth, increases progressively during adult life, and becomes very marked in old age. If it should restrain cell proliferation *in vivo* as much as *in vitro*, its rôle in many physiological and morbid processes would be important. Therefore, an investigation of its nature was begun. The purpose of the experiments described in this article was to study the modifications occurring in the rate of growth of fibroblasts when the serum composing the culture medium had been heated at various temperatures.

EXPERIMENTAL.

The serum was obtained from the plasma of chickens about 1 year old which had fasted for 24 hours. Its inhibiting action was determined by comparing the rate of growth of fibroblasts in a medium containing 50 per cent serum and in another containing 50 per cent Tyrode solution. The growth index of this serum averaged 0.9, which indicated that the inhibiting power was slight. The serum was used in the preparation of the medium in two different concentrations. When the fibrin of the coagulum was obtained from plasma, the medium was composed of 33.3 per cent plasma, 4 per cent tissue juice, and 62.6 per cent serum. When fibrinogen was used instead of plasma, the medium was made of 20 per cent fibrinogen suspension,²

¹ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

² Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

50 per cent serum, 27.5 per cent Tyrode solution, and 2.5 per cent tissue juice.

The action of unheated serum was compared with that of heated serum. Serum heated at 56°C. for a period of time varying from $\frac{1}{2}$ to 24 hours became slightly opalescent. When it was heated at 70°C. for $\frac{1}{2}$ hour, a precipitate occurred which was thrown down by centrifugation. The supernatant fluid was transparent, slightly opalescent, and not very viscous. The serum heated at 100°C. coagulated. The fluid which remained around the coagulum was used in the preparation of the medium.

The fibroblasts were taken from stock cultures of a 9 year old strain of connective tissue, and the cultures prepared in the usual manner. They were allowed to grow for 48 hours and the measurements were taken by outlining the original fragment and the new tissue under the projection apparatus and determining the area with the planimeter. The relative increase was calculated.³

1. *Action of Serum Heated at 56°C.*—In the first series of experiments, a comparison of the action of sera unheated and heated at 56°C. for 1 hour was made in media containing 33.3 per cent chicken plasma (Tables I and II). The rate of growth of fibroblasts in both media was identical.

In a second series of experiments, 20 per cent fibrinogen suspension was used instead of plasma. Differences in the rate of growth under the influence of heated and unheated sera became apparent (Tables III to VIII). The time during which the serum was kept at the temperature of 56°C. had little influence on its inhibiting action. The rate of growth of fibroblasts in sera heated at 56°C. for $\frac{1}{2}$ to 24 hours was decreased about 15 per cent.

2. *Action of Serum Heated at 70°C.*—In the first series of experiments, the action of serum heated at 70°C. for $\frac{1}{2}$ hour was compared with that of serum heated at 56°C. (Table IX). The rate of growth in the serum heated at 70°C. was 22 per cent slower than in the serum heated at 56°C.

In a second series of experiments, the action of sera unheated and heated at 70°C. was compared (Table X). The growth was found to

³ Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

TABLE I.

Rate of Growth of Fibroblasts in Plasma and Serum, Unheated and Heated at 56°C.

Experiment No.	Culture No.	Width of ring.	
		Serum 62.6 per cent.	
		Unheated.	Heated.
1	23191	5.2	4.9
2	23192	4.9	5.1
3	23193	4.6	4.7
Average		4.9	4.9
4	1185-1, 1185-2	5.0	4.5
5	1185-3, 1185-4	4.0	4.0
Average		4.5	4.25
6	23194	5.0	4.5
7	23195	4.0	4.5
8	23196	5.0	5.0
Average		4.7	4.7

The serum used in the first three experiments was heated at 56°C. for 1 hour, in the remaining five experiments, for 2 hours. The rate of growth is expressed by the width of the ring of new tissue.

TABLE II.

Rate of Growth of Fibroblasts in Plasma and Serum, Unheated and Heated at 56°C.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Serum 62.6 per cent.		
		Unheated.	Heated.	
1	23191	4.08	4.02	0.99
2	23192	3.76	3.91	1.04
3	23193	3.46	4.05	1.17
Average				1.07
4	23194	3.85	3.61	0.94
5	23196	3.82	4.07	1.07
Average				1.00

In the first group of experiments, the serum was heated at 56°C. for 1 hour, in the second group, for 2 hours. The rate of growth is expressed by the relative increase of the tissue.

TABLE III.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for ½ Hr.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Serum 50 per cent.		
		Unheated.	Heated.	
1	20936	3.36	2.72	0.80
2	20937	2.13	1.62	0.76
3	20938	3.50	2.68	0.77
4	21491	5.06	4.87	0.96
5	21492	3.43	2.93	0.85
6	21493	4.80	3.43	0.71
7	21450	2.76	2.51	0.91
8	21841	2.90	2.20	0.76
9	21842	2.60	2.40	0.92
10	21843	2.80	2.30	0.82
11	21844	2.40	2.40	1.00
12	23110	2.16	1.89	0.88
Average		3.16	2.66	0.85

TABLE IV.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 1 Hr.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Serum 50 per cent.		
		Unheated.	Heated.	
1	23164	3.72	3.42	0.92
2	23165	3.73	3.37	0.90
3	23166	2.91	2.91	1.00
4	23167	2.48	2.30	0.93
5	23226	3.55	4.00	1.12
6	23227	4.35	4.47	1.03
7	23246	2.28	1.81	0.79
8	23248	2.74	2.00	0.73
9	23249	2.21	1.85	0.84
Average.....				0.92
10	23477	5.12	4.64	0.91
11	23478	3.92	3.55	0.91
12	23479	3.80	3.12	0.82
13	23944	2.81	2.22	0.79
14	23945	3.20	2.65	0.83
15	23946	3.14	2.56	0.82
Average.....				0.85

In the first nine experiments, 5 per cent embryo juice was used, and in the remaining six experiments, 2.5 per cent embryo juice.

TABLE V.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 2 Hrs.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Serum 50 per cent.		
		Unheated.	Heated.	
1	23168	3.12	2.77	0.89
2	23169	3.00	2.74	0.91
3	23170	3.19	2.67	0.84
4	23171	3.10	2.83	0.91
5	23228	3.50	3.22	0.92
6	23229	3.93	3.80	0.97
7	23251	3.24	2.85	0.88
8	23252	2.55	2.33	0.91
9	23253	3.32	3.00	0.90
Average				0.90

In the first two experiments, 5 per cent embryo juice was used, and in the remaining seven experiments, 2.5 per cent embryo juice.

TABLE VI.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 6 Hrs.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Serum 50 per cent.		
		Unheated.	Heated.	
1	23288	3.21	2.70	0.84
2	23289	4.21	3.20	0.76
3	23290	4.05	3.21	0.79
Average				0.80

TABLE VII.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 12 Hrs.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Serum 50 per cent.		
		Unheated.	Heated.	
1	23291	3.50	2.78	0.79
2	23292	3.70	3.11	0.84
3	23293	3.67	3.22	0.88
Average				0.84

TABLE VIII.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 56°C. for 24 Hrs.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Serum 50 per cent.		
		Unheated.	Heated.	
1	23440	3.70	3.08	0.83
2	23441	3.58	2.93	0.82
3	23442	3.87	3.03	0.78
4	23334	3.07	2.90	0.94
5	23335	3.26	3.15	0.96
6	23336	3.48	2.90	0.83
Average				0.86

TABLE IX.

Rate of Growth of Fibroblasts in Serum Heated at 56°C. for $\frac{1}{2}$ Hr. and at 70°C. for $\frac{1}{2}$ Hr.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Serum heated at } 70^{\circ}\text{C.}}{\text{Serum heated at } 56^{\circ}\text{C.}}$
		Serum 50 per cent.		
		Heated at 56°C.	Heated at 70°C.	
1	23812	2.70	1.98	0.73
2	23813	2.33	2.00	0.86
3	23814	3.06	2.56	0.84
4	23846	2.70	2.18	0.81
5	23847	2.56	1.92	0.75
6	23848	2.76	2.08	0.75
7	23886	3.26	2.76	0.85
8	23887	2.67	2.37	0.89
9	23888	3.00	2.20	0.73
10	23941	3.40	2.44	0.72
11	23942	3.40	2.43	0.71
12	23943	3.78	2.80	0.74
13	23947	2.39	1.74	0.73
14	23948	2.33	1.81	0.78
15	23949	2.84	2.28	0.80
Average				0.78

TABLE X.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 70°C. for $\frac{1}{2}$ Hr.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$
		Serum 50 per cent.		
		Unheated.	Heated.	
1	23337	3.20	2.28	0.71
2	23338	4.33	3.14	0.73
3	23339	4.24	2.69	0.63
4	23443	3.91	2.08	0.53
5	23444	3.43	1.69	0.49
6	23445	3.95	1.69	0.43
7	23709	3.62	2.25	0.62
8	23710	3.90	2.57	0.66
9	23711	3.90	2.60	0.67
10	23679	2.38	1.94	0.82
11	23680	2.21	1.80	0.81
12	23681	3.88	2.93	0.76
Average				0.66

be 34 per cent slower in heated than in unheated serum. Both series of experiments showed almost identical results. It must be observed also (Tables IX and X) that in both series the values for the relative increase of the tissues in serum heated at 70°C. are about similar. The consistency of the results demonstrates that there were no technical errors.

TABLE XI.

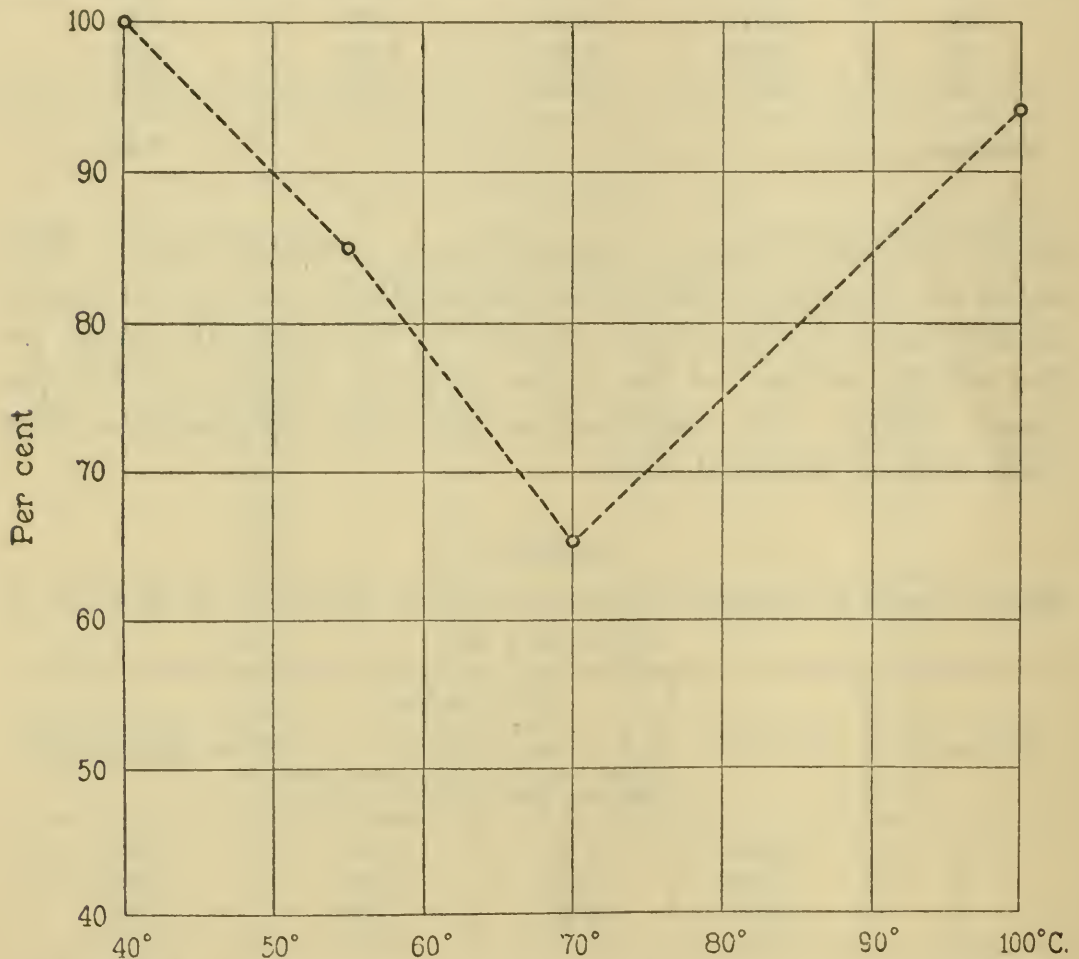
Rate of Growth of Fibroblasts in Serum Heated at 100°C. for 10 Min. and at 70°C. for $\frac{1}{2}$ Hr.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated at } 70^{\circ}\text{C.}}{\text{Heated at } 100^{\circ}\text{C.}}$
		Serum 50 per cent.		
		Heated at 100°C.	Heated at 70°C.	
1	23889	3.35	2.30	0.69
2	23890	2.60	1.90	0.73
3	23891	3.26	2.55	0.78
Average				0.73

TABLE XII.

Rate of Growth of Fibroblasts in Serum, Unheated and Heated at 100°C. for 10 Min.

Experiment No.	Culture No.	Relative increase.		Ratio: $\frac{\text{Heated serum}}{\text{Unheated serum}}$.
		Serum 50 per cent.		
		Unheated.	Heated.	
1	23632	3.12	3.06	0.98
2	23633	3.43	3.01	0.88
3	23634	3.19	3.02	0.95
4	23635	2.88	2.85	0.99
5	23654	2.91	2.87	0.99
6	23655	3.05	3.44	1.13
7	23656	3.32	3.14	0.94
8	23657	3.30	2.90	0.88
9	23849	4.03	3.25	0.81
10	23850	3.16	2.74	0.87
11	23851	3.81	3.56	0.93
Average				0.94



TEXT-FIG. 1. Variations in the rate of growth of fibroblasts in function of the temperature at which the serum was heated. The rate of growth of fibroblasts in unheated serum was considered to be equal to 100 per cent.

3. *Action of Serum Heated at 100°C.*—The fluid remaining after coagulation was compared with serum heated at 70°C. and with unheated serum. The figures show that the rate of growth in serum heated at 100°C. was 27 per cent faster than in serum heated at 70°C.; that is, about equal to the growth rate in unheated serum (Table XI). The experiments in which a comparison was made between the respective actions of unheated serum and serum heated at 100°C. confirmed these results (Table XII). The amount of growth was practically identical in both unheated serum and serum heated at 100°C. It must be remembered that differences equal to or smaller than 10 per cent may be due to experimental errors.

A curve was plotted expressing the variations of the rate of growth of the fibroblasts in function of the temperature at which the serum had been heated (Text-fig. 1). It shows that the rate of cell proliferation decreased after the serum had been heated at 56°C., and became still slower after it had been heated at 70°C. A marked increase in the rate of growth took place after the serum had been heated at 100°C.

DISCUSSION.

The results obtained in the preceding experiments may be summarized as follows:

The action of heat at 56°C. increased by 15 per cent the inhibiting action of serum obtained from young adult chickens on the proliferation of fibroblasts. The action of heat at 70°C. increased the inhibiting action by 34 per cent. When the serum had been heated at 100°C., its inhibiting action became about equal to that of non-heated serum. Therefore, heated serum contained a factor which markedly inhibited the growth of fibroblasts and which developed at, or resisted a temperature of 70°C.

These experiments confirmed the results obtained by Ingebrigtsen⁴ in his study of the growth of guinea pig bone marrow in homologous serum unheated and heated at 56°C. The growth of bone marrow was found to be more extensive in unheated than in heated serum. The differences in the action of both sera were more striking than in

⁴ Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 397.

our experiments. This was due to the fact that Ingebrigtsen used a medium containing a very large amount of serum and observed lymphocytes instead of fibroblasts.

The increase of the inhibiting power of serum after it had been heated at 56° and 70°C. may be considered as due to the production, under the influence of heat, of a change which renders the serum more toxic for the homologous fibroblasts. But it may also be attributed to the destruction of substances presenting the same heat resistance as complement and amboceptor, and partly protecting the cells against the inhibiting action of a third substance resisting heat at 70°C. Serum modified by heat acts in an opposite manner on heterologous tissues. Heated serum is a better culture medium for heterologous cells than unheated serum, as has already been shown by Ingebrigtsen.⁴ We have found lately that the inhibiting action of dog, rabbit, and cat serum, heated at 56° and 66°C., on the rate of multiplication of fibroblasts is very much decreased. It seems that the factors which protect the organism against foreign cells and bacteria might also oppose the growth-inhibiting factor of serum and allow the cells to display a greater activity.

CONCLUSIONS.

The inhibiting action of homologous serum on the proliferation of fibroblasts *in vitro* was increased after the serum had been heated at 56° and 70°C. This action decreased after the serum had been heated at 100°C.

CICATRIZATION OF WOUNDS.

XIII. THE TEMPERATURE COEFFICIENT.

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(Received for publication, January 11, 1922.)

INTRODUCTION.

Cicatrization is a complex phenomenon which probably requires the coordinated activity of many factors. Although it may be expressed by a simple formula,¹ its mechanism involves several different agencies. The formation of granulating tissue during the latent period,² its contraction, the mobilization and proliferation of the epithelium, and the wandering of the latter on the surface of the granulations are themselves intricate processes, which may be governed by physical as well as chemical changes. Alterations of the viscosity of the surface tension of the fluids and the anatomical structures may play as important a rôle as chemical transformations. In order to ascertain whether physical or chemical changes are more especially involved, the value of the temperature coefficient of the phenomenon was measured.

EXPERIMENTAL.

The experiments were performed on alligators because these animals may be kept as well at a temperature of 38° as at 23°C. Two young alligators were used, their weights being respectively 309 and 72 gm. A rectangular flap of skin on the ventral surface of the body was resected with a sharp knife and the outline of the wound traced with India ink upon a piece of cellophane placed over the surface. The tracings were transferred to paper and the areas measured with a

¹ du Noüy, P. L., *J. Exp. Med.*, 1919, xxix, 329.

² Carrel, A., *J. Exp. Med.*, 1921, xxxiv, 425.

planimeter. The animals were placed in a room having a temperature of 38°C. until the wounds had healed. Several days later a second resection of skin was made in a different area on the ventral surface of the body of each animal, as nearly as possible the same size as the first. This was done by transferring the outline of the previous tracing to the skin surface and then excising the flap. The animals were placed in a room, the temperature of which averaged 23°C.

The wounds could not be protected by a dressing. They were necessarily slightly infected, and a membrane or scab formed at the surface. Under this covering, cicatrization was taking place, but its progress could not be followed closely. To overcome this hindrance to precise observation, the edges of the scab were gently and progressively loosened with the point of a knife and a pair of small thumb forceps, and then the extent of the formation of the epithelial layer under the scab could be seen. The membrane was easily loosened from the portion of the wound which had already healed, while the

TABLE I.

Experiment No.	Weight of animal.	Temperature.	Area of wound.	Length of time required for cicatrization.	Temperature coefficient for 10°C.
	<i>gm.</i>	<i>°C.</i>	<i>sq. cm.</i>	<i>days</i>	
1	309	38	1.2	11	2.36
	314	23	1.3	29	
2	72	38	0.4	8	1.88
	75	23	0.5	19	

scab proved adherent wherever epithelization had not occurred. If an attempt were made to remove the protecting membrane, a slight hemorrhage started. As such a traumatism delays the process of healing, observations had to be made with the greatest care. Errors of 1 or 2 days in the time of cicatrization may have occurred on account of the technical difficulties. Two experiments were made on each animal, one at a temperature of 38°C., and the other at 23°C. (Table I).

In the first experiment, the temperature coefficient obtained, $\frac{K_1}{K_0}$, is equal to 2.47, and in the second to 1.90, for a difference of

15°C., between 38° and 23°C. The difference between the values of these two coefficients may be attributed to experimental errors, but is not of a greater magnitude than those observed in similar phenomena. Their value for a range of 10°C. (between 20° and 30°C.) was obtained by the formula quoted by Robertson:³

$$\frac{K_1}{K_o} = e^{\frac{\mu}{2} \left(\frac{T_1 - T_o}{T_1 T_o} \right)}$$

In the first case, it is equal to 2.36, and in the second to 1.88 for the same interval. The mean value is 2.12.

DISCUSSION AND CONCLUSIONS.

For a rise of temperature of 10°C., the rate of cicatrization was increased about twofold. This result could be expected, since wound healing is closely related to the phenomenon of growth and regeneration. It is classical that changes in temperature affect the metabolism⁴ and the development of certain organisms⁵ in the same manner as a chemical reaction. In spite of the complexity of the factors which bring about the cicatrization of a wound, it appears that the velocity of the phenomenon depends on the rate at which certain chemical changes take place.

³ Robertson, T. B., Principles of biochemistry, for students of medicine, agriculture and related sciences, Philadelphia, 1920, 419.

⁴ Krogh, A., *Z. allg. Physiol.*, 1914, xvi, 178.

⁵ Hertwig, O., *Arch. mikr. Anat.*, 1898, li, 319. Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1917, iii, 384. Moore, A. R., *Arch. Entwcklgsmechn.* 1910, xxix, 146.

ACTION OF ANTIGEN ON FIBROBLASTS IN VITRO.

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It is known that tissue cells cultivated outside of the organism are able to produce antibodies against a given antigen when it is added to the medium. Goat red blood corpuscles, added to the medium in which guinea pig bone marrow was cultivated, determined the production of hemolysins by the guinea pig cells.¹ In the experiments described in this paper, the action of an antigen on the rate of proliferation of fibroblasts was investigated. An attempt was made to learn whether one part of a fragment of culture would grow at the same rate as the other part, when a small amount of a foreign protein was added to the medium for a long period of time; and whether a change in their respective rates of growth would occur if the fibroblasts cultivated in homogenic plasma, and in the same plasma containing a small amount of a heterogenic protein, were transferred into a medium containing this latter protein under a high concentration.

Technique.

The tissues used in these experiments were taken from a 9 year old strain of fibroblasts.² The cultures were prepared in the usual way, incubated for 48 hours, measured by projection of the area of growth, and transferred to a fresh medium. The foreign protein used was human ascitic fluid; in a few experiments dog serum was used. The culture which was selected for the experiment was divided into two equal parts. One of the fragments was placed in a medium consisting of equal volumes of chicken plasma and fresh chick embryo juice. The other was placed in a medium containing equal volumes of plasma

¹ Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

² Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

and embryonic tissue juice to which had previously been added about 14 per cent of ascitic fluid, making the percentage of foreign protein in the total culture medium about 7 per cent. Care was taken to avoid contaminating the control culture by instruments, knives, and needles which had been in contact with the foreign protein. When transferred to a new medium, each of the two cultures was divided. One half was used for continuing the experiment, and the other half was discarded, unless this part was used as a test to see how the cultures would act when exposed to the foreign protein in a high concentration.

RESULTS.

The rate of growth of the two strains of a complete experiment of nineteen passages during a period of about 40 days is recorded in Table I and Text-fig. 1. At the beginning, the rate of growth of both cultures showed a slight difference. The culture to which the small amount of ascitic fluid was added nearly always showed a slightly higher rate of growth than the control culture. This might be due to secondary causes. The fluctuations of the rate of proliferation are due, to some extent, to the influence of slight changes in temperature of the incubator and similar periodical causes. When the control showed a high rate of growth, the experiment showed a lower rate, and *vice versa*, but after about eight or ten passages both cultures showed fluctuations in the same direction. As a whole, no marked differences between the two rates of growth seem to take place.

When an attempt was made to see how both strains would act when exposed to a high concentration of the protein used for immunization, subcultures were made of the control (non-immunized strain) and of the immunized strain, and transferred into a medium composed of plasma, embryonic tissue juice, and ascitic fluid. The amount of ascitic fluid in the subcultures was 50 per cent or more, and exerted a marked inhibiting influence on the rate of growth of a normal culture. The rate of growth of the control cultures which had not been exposed to ascitic fluid decreased markedly and was even followed by death (Text-fig. 2). At Passages 7, 10, 12, 14, and 17, half the tissue fragment was cultivated in a medium containing about 50 per cent of ascitic fluid, while the other half was used for the continuation of the

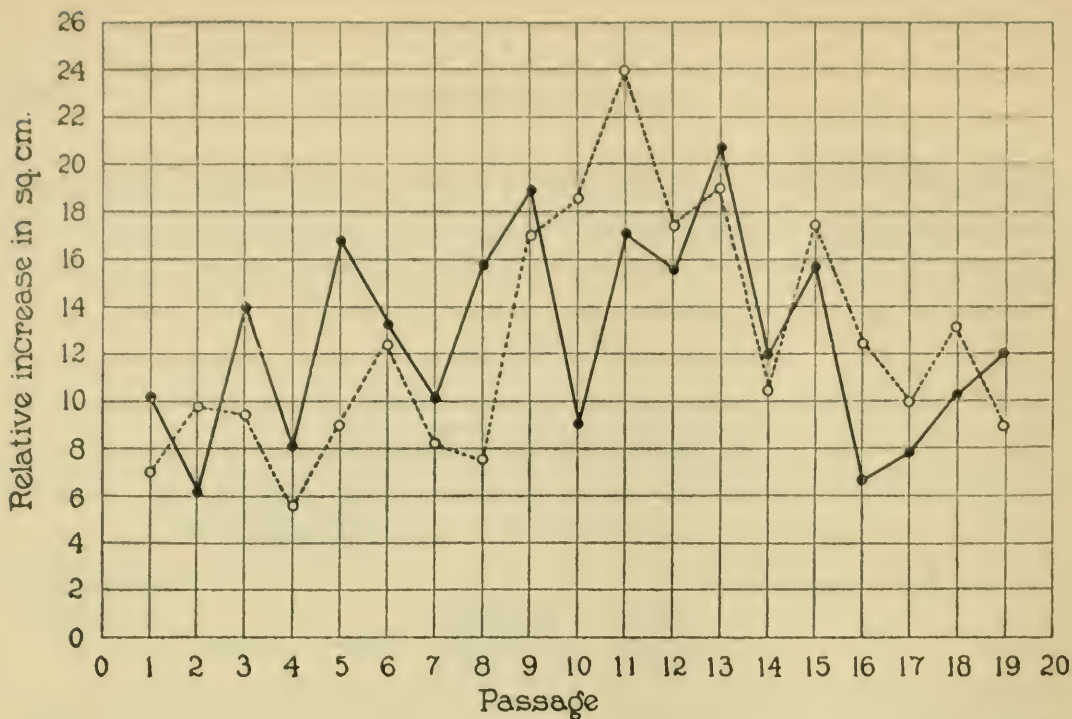
strain. The dots *A*, *B*, *C*, *D*, and *E* represent the rate of growth of these subcultures in 48 hours (Text-fig. 2.) It is shown that 50 per cent ascitic fluid has a marked inhibiting influence on the rate of

TABLE I.

Rate of Growth of the Immunized and Non-Immunized Strains of Fibroblasts for a Period of Nineteen Passages.

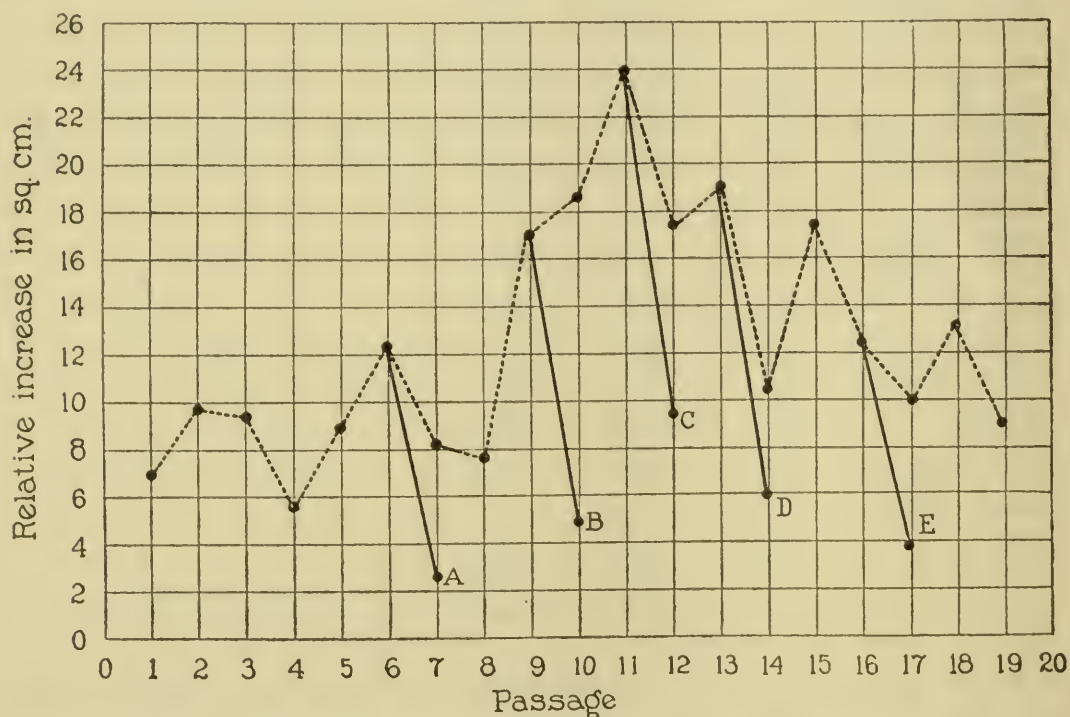
Culture No.	Passage No.	Relative increase.		Relative increase of subcultures in 50 per cent ascitic fluid.		Ratio $\left(\frac{E}{C}\right)$ of subcultures.
		Control; homogenic medium.	Experiment; homogenic medium with 7 per cent ascitic fluid.	Control.	Experiment.	
537-1, 537-2	1	7.0	10.2			
549, 550	2	9.8	6.2			
559, 560-1	3	9.4	14.0			
571, 572	4	5.6	8.2			
585, 586	5	9.0	16.8			
599, 600	6	12.4	13.4			
610-1, 610-2 611-1, 611-2	7	8.2	10.2	2.8	13.8	4.9
623, 624	8	7.6	15.8			
642, 643	9	17.0	19.0			
659-1, 659-2 660-1, 660-2	10	18.6	9.2	4.6	16.2	3.5
672, 673	11	23.9	17.2			
686-1, 686-2 687-1, 687-2	12	17.4	15.6	9.4	23.2	2.4
702, 703	13	19.0	20.8			
718-1, 718-2 719-1, 719-2	14	10.4	12.0	6.0	23.0	3.4
733, 734	15	17.4	15.8			
749, 750	16	12.4	6.8			
760-1, 760-2 761-1, 761-2	17	10.0	8.0	3.8	8.4	4.2
775, 776	18	13.2	10.4			
799, 800	19	9.0	12.2			

growth of a normal culture of fibroblasts. The rate of growth of the fibroblasts in a culture medium which always contained about 7 per cent ascitic fluid is shown in Text-fig. 3. At Passages 7, 10, 12, 14, and 17, subcultures were made in a medium containing about 50 per

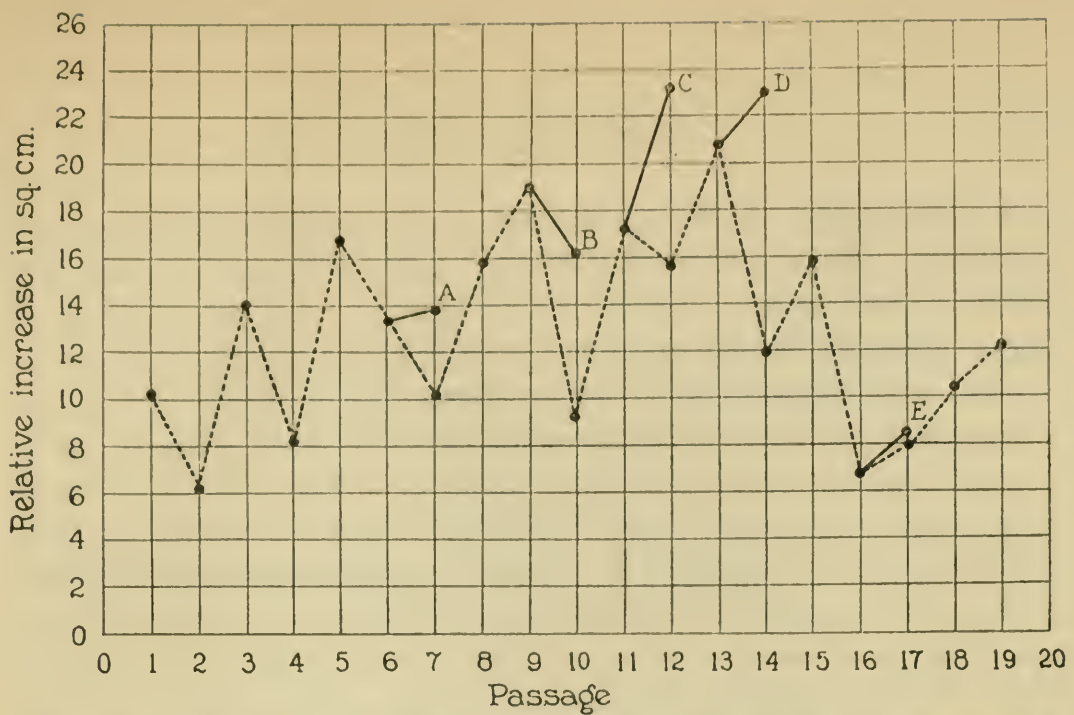


In all the figures the ordinates represent the relative increase in square centimeters and the abscissæ the number of passages at 48 hour intervals.

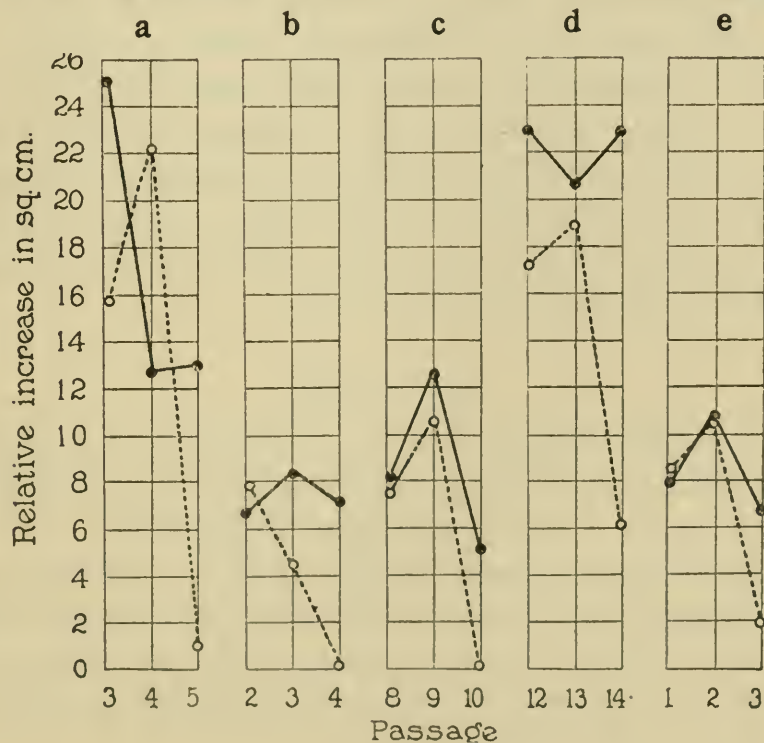
TEXT-FIG. 1. The rate of growth of two cultures of fibroblasts, the control (dotted line) cultivated in the usual homogenic culture medium, the other (solid line) in the same medium containing 7 per cent ascitic fluid.



TEXT-FIG. 2. The rate of growth of the non-immunized strain and subcultures. The dotted line represents the rate of growth of fibroblasts in homogenic culture medium. The solid lines (A, B, C, D, and E) represent the rate of growth of the subcultures of the non-immunized strain in a medium containing 50 per cent ascitic fluid.



TEXT-FIG. 3. The rate of growth of the immunized strain and subcultures. The dotted line represents the rate of growth of fibroblasts in the culture medium containing 7 per cent ascitic fluid. The solid lines (A, B, C, D, and E) represent the rate of growth of the subcultures of the immunized strain in a medium containing 50 per cent ascitic fluid.



TEXT-FIG. 4, a to e. (a to d) The rate of growth of the non-immunized (dotted line) and the immunized (solid line) strain of fibroblasts. Of the three passages shown, the last one represents the rate of growth in the culture medium containing 50 per cent ascitic fluid. (e) The rate of growth of two strains of fibroblasts, one (solid line) cultivated in a medium containing 7 per cent dog serum, the other (dotted line) in a medium containing no foreign protein. The last passage shows the growth of both strains in the culture medium containing 50 per cent dog serum.

cent ascitic fluid, and the dots *A*, *B*, *C*, *D*, and *E* indicate the rate of growth. It may be seen that the rate of growth increased markedly instead of decreasing, as in the control cultures. There was always a sharp difference in the response of the two cultures to the same amount of foreign protein. It was certain that one of the strains had become adapted to the presence of the foreign protein, while the other was still subject to its inhibiting influence. The same phenomena were observed in several other experiments and are expressed by Text-fig. 4, *a* to *e*. In Text-fig. 4, *a*, the curve shows the rate of growth during two passages in 7 per cent foreign protein, and after exposure to 50 per cent foreign protein. The non-immunized strain almost died. Its rate of growth decreased from 22 sq. cm. to about 1 sq. cm., while the immunized strain grew at the usual rate, as if nothing had happened. In another experiment (Text-fig. 4, *b*), the non-immunized strain was killed, and the immunized strain not affected at all. The same phenomenon is shown in Text-fig. 4, *c* and *d*.

In a few experiments, dog serum which had had a strong inhibiting influence on the growth of fibroblasts was used as antigen. The result showed the same action as with ascitic fluid (Text-fig. 4, *e*). The immunized strain was cultivated in a medium containing 7 per cent dog serum, and the control in a medium containing no foreign protein. For the final test, both strains were placed in a medium containing 50 per cent dog serum; the rate of growth of the control decreased much more than that of the immunized strain.

CONCLUSIONS.

It may be concluded that, under the conditions of the experiments:

1. A small amount of foreign protein added to the culture medium does not modify the rate of proliferation of fibroblasts.
2. A large amount of foreign protein added to the culture medium decreases markedly the rate of proliferation of fibroblasts cultivated previously in homogenic medium, while it does not decrease the rate of proliferation of fibroblasts cultivated previously in the presence of a small amount of the foreign protein.
3. Fibroblasts *in vitro* respond to the presence of an antigen in the culture medium by becoming immunized against its action.

THE VASELINE TUBE AND SYRINGE METHOD OF MICRO GAS ANALYSIS OF BACTERIAL CULTURES.

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PLATES 55 TO 57.

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The methods to be described have been evolved not so much because of dissatisfaction with results which could be obtained by methods previously used but rather for reasons of greater convenience and economy. It is for these reasons that the Smith fermentation tube has continued in use long after its limitations have been recognized. The Smith tube was never recommended as a means for accurate study of the gas-forming function of bacteria. It was recommended as an instrument for collecting and approximately measuring and analyzing the gases which are given off by certain cultures when grown in this tube. The result of such measurement and analysis was found to be a means of distinguishing certain broad groups of bacteria. The gas was produced under the conditions imposed by the tube and the gas formula $\left(\frac{\text{H}_2}{\text{CO}_2}\right)$ was understood to be valid for those conditions only. Critics of the Smith fermentation tube should not lose sight of these facts.

More efficient and more elaborate forms of apparatus have been devised by various authors with a different purpose in view, namely to collect all the gas which a culture forms, to measure both that given off from the medium and that held by the medium, and to make very accurate analyses of these gases. I refer particularly to the apparatus described by Keyes (1909), Friebert (1913, *b*), Rogers, Clark and Davis (1914), and Wolf and Harris (1917). These methods are much better adapted for the careful study of the gas-forming activity of a

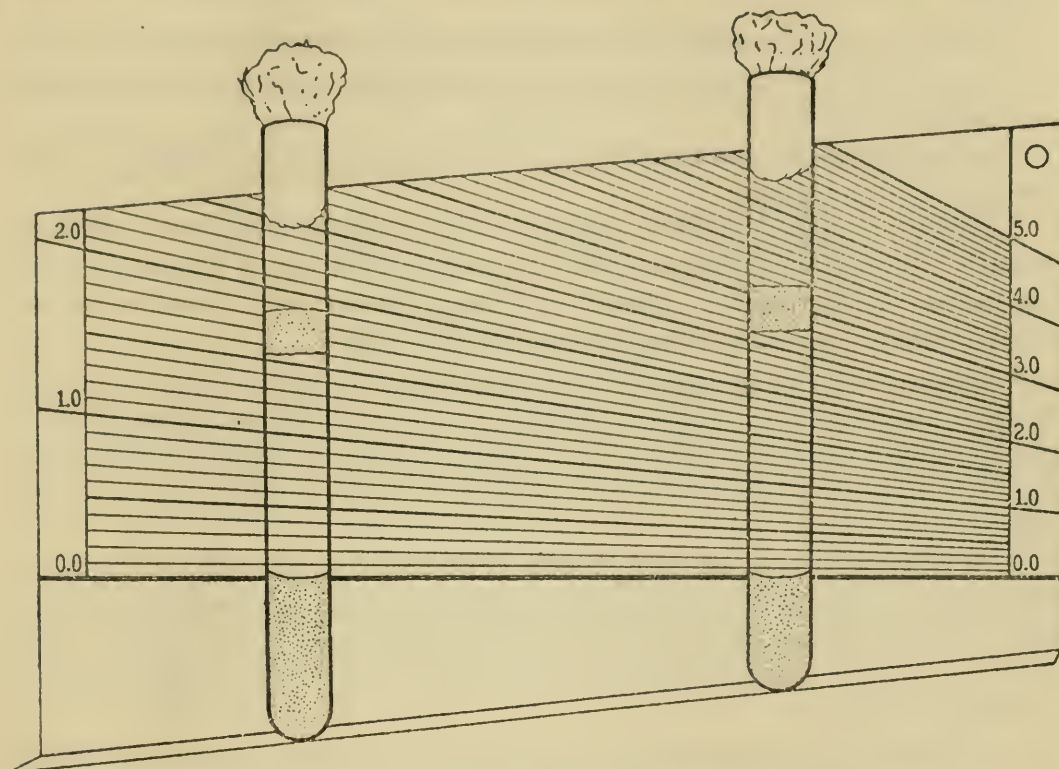
few cultures than for the comparative study of a large number of strains. Doubtless the apparatus described by Van Slyke and Stadie (1921) could be used for bacterial cultures and should yield more accurate results than the syringe. However, it is believed that the method to be described retains the simplicity of the Smith tube, at least something of the greater accuracy of the more elaborate methods, and some advantages for the bacteriologist possessed by none of the older methods.

During a study of anaerobic bacteria still in progress, use was made of small amounts of media covered by vaseline in test-tubes. It was noticed that as gas was formed by a culture the vaseline was forced up the tube, the vaseline plug remaining perfectly intact and acting as a self-lubricating piston at the temperature of the room or incubator. We have continued to use this form of culture tube in the manner to be described.

Measurement of Gas above the Medium.

A record of the amount of gas present above the medium may be made at any time by marking with a wax pencil on the side of the tube the lower level of the vaseline plug. To measure the amount of gas in the tube a device somewhat like the Frost gasometer for the Smith fermentation tube is used. The gasometer shown in Text-fig. 1 is made of sheet tin or brass. The lower edge is turned at a right angle towards the observer so that a shelf is formed on which rests the bottom of the tube being examined. The base-line of the graduations ruled on the surface of the gasometer is shown as a double line marked 0.0 and placed diagonally with reference to the shelf and above it. The culture tube is pushed along the shelf until the meniscus of the culture medium rests at the base-line. The graduations above the base-line radiate from a point on the base-line at some distance to the right of the figure so that when the tube cuts the base-line vertically the amount of gas between the medium and the vaseline plug can be read as volumes and tenths of a volume of the amount of medium present in the tube, or, if multiplied by 100, as volumes per cent. If the culture produces sufficient gas to force the vaseline up nearly to the cotton plug the amount of gas is recorded, then by directing the flame of a micro burner against the side of the tube the vaseline is

melted and allowed to drop down onto the surface of the medium and to solidify again. This may be done repeatedly in which case the total gas formed above the medium is obtained by addition of the measurements recorded before each melting of the vaseline plug.



TEXT-FIG. 1. The gasometer with two tubes in position for the measurement of the gas above the medium in terms of the volume of medium employed.

Determination of CO₂ in the Gas above the Medium.

By means of a Luer tuberculin syringe with a long fine needle¹ attached by means of a short length of capillary rubber tubing a sample of gas may be withdrawn and the CO₂ determined in the syringe. The technique and necessary equipment are illustrated in Figs. 1 and 2. The various steps of the technique are as follows:

¹ The needle used was Gauge 20 and 6 inches long, specially made for us by Becton, Dickinson and Co., Rutherford, N. J. There is also needed a small adapter such as that listed by Arthur H. Thomas Co., No. 9419, for 606 Hose End to Luer Slip Needles.

1. Rinse the syringe and needle with dilute acid which is then expelled as completely as possible by forcing air into and out of the syringe a number of times. Push the syringe plunger in as far as it will go or, better, to an exact reading at the lower end of the scale on the barrel of the syringe.

2. Dry and sterilize the needle by passing it through the flame a number of times.

3. With the usual precautions to maintain sterility draw the cotton plug from the culture tube and replace it with the needle held between the cotton plug and the side of the tube.

4. Push the needle down until the point passes through the vaseline plug and well into the zone of gas.

5. With the syringe held as shown in Fig. 1 slowly withdraw a measured quantity of gas (something less than 1 cc. so that when step 7 is taken the total gas can be read between the plunger and the meniscus). The vaseline plug will be seen to move down the tube as the gas is withdrawn.

6. Leave the cotton plug in place and pull the needle up until the point remains in the upper part of the vaseline. With a small hot spatula touch the outside of the test-tube opposite the track of the needle in the lower part of the vaseline plug. Leave the spatula in contact with the tube just long enough to close the opening in the vaseline.

7. Withdraw the needle from the tube, immediately stick the end into the dilute acid, and with the syringe held vertically draw up acid until the meniscus can be seen and read against the scale on the lower end of the syringe. Read the exact amount of gas (and air) in the syringe between the plunger and the meniscus of acid.

8. Stick the end of the needle into a 2 or 3 per cent solution of sodium hydroxide. With the apparatus in the position shown in Fig. 2 draw out the plunger until the alkali begins to enter the barrel of the syringe and neutralizes the acid there. As carbon dioxide is absorbed more alkali is automatically drawn into the syringe without further use of the plunger. With the end of the needle still in the alkali solution rock the syringe back and forth a number of times to insure complete absorption of the CO_2 .

9. Again hold the syringe vertically with the plunger uppermost and read the amount of residual gas in the syringe. The difference between this reading and the one taken in step 7 equals the amount of CO_2 in the sample taken. The percentage of CO_2 in the sample is easily calculated and may be expressed as a gas ratio $\left(\frac{\text{H}_2}{\text{CO}_2}\right)$ if so desired.

We have tried a number of slight modifications of the details of the above technique but the above is probably the most perfect. The accuracy of the method is apparently limited only by the fineness of the graduations on the syringe. These should be at most 0.01 cc.

We are able to get almost as accurate results with 0.1 cc. of gas sample as with 1.0 cc. It is possible to determine the CO_2 content of a good sized bubble beneath the vaseline plug or in an agar shake culture. It is an advantage to select a tuberculin syringe having the following characteristics: (a) finely cut graduations, (b) a long slender barrel with considerable space above the graduations, (c) a well fitting plunger of colored glass with a sharp square-cut end which can be accurately read against the graduations on the colorless glass barrel. The carbon dioxide content of the small amount of atmospheric air contained in the needle and capillary rubber tube is a negligible quantity well within the limits of error. If the cultures are allowed to cool down to room temperature before the gas analysis is made and if the acid and alkali solutions are kept at the same temperature the temperature factor is also negligible. Formerly we took the precaution of allowing the end of the needle to be sealed by the vaseline (melted by the hot spatula) as it was withdrawn from the tube and then pricking this seal just as the needle was placed into the acid but it has been found unnecessary. There is no appreciable interchange of air and gas through the needle during the short time it is exposed to the air while being transferred from the culture tube to the dilute acid.

Determination of Carbonates and CO_2 in the Medium.

The carbonates and CO_2 in a fluid medium may be determined by means of the same apparatus. The equipment and technique are illustrated in Fig. 3. The steps of the technique are as follows:

1. Rinse the syringe and needle thoroughly with distilled water.
2. Dry and sterilize the needle in the flame.
3. With aseptic precautions place the needle so that it is held between the cotton plug and the side of the tube as for the determination of CO_2 above the medium.
4. Having placed the end of the syringe plunger at one of the graduation marks on the lower end of the barrel push the needle down the side of the tube through the vaseline plug (if vaseline is used) until the point is in the medium.
5. Draw the plunger of the syringe back just 0.1 cc.
6. Withdraw the needle with the same precautions as described in step 6 for the determination of CO_2 above the medium.
7. Draw into the needle about 0.03 cc. of air.

8. Stick the point of the needle into a small vial of capryl alcohol (colored with scarlet R) and draw 0.01 cc. into the needle, followed by 0.01 cc. of air.

9. Draw into the needle 0.05 cc. of 5 per cent sulfuric acid (colored with methyl red).

10. Holding the syringe vertically with the open needle uppermost draw back the plunger just until all of the red capryl alcohol and sulfuric acid are seen to be within the barrel of the syringe.

11. With the index finger of the left hand tightly stop up the end of the syringe where the rubber tube is attached and holding it as shown in Fig. 3 draw down the plunger while with a partial vacuum within the syringe the air leaks by the plunger and passes up through the contents of the syringe in a stream of fine bubbles. When the end of the plunger reaches a position a little beyond the graduations it should be allowed to remain here until the air ceases bubbling.

12. Turn the syringe to a vertical position with the plunger uppermost, release the finger from the rubber tube, push in the plunger to the beginning of the graduation marks, wait a few seconds to allow the fluid to drain down the sides of the syringe, and read the amount of gas and air present between the meniscus and the plunger.

13. Without changing the position of the syringe, needle hanging down, expel most of the fluid until the meniscus reaches the bottom of the syringe. Without admitting any air dip the end of the needle, now filled with fluid, into the sodium hydroxide solution and with the syringe held in the position shown in Fig. 2 draw up some of the alkali. Rock the syringe back and forth a few times. Again expel most of the fluid, holding the syringe in a vertical position and taking care to expel none of the gas. Draw in a fresh portion of sodium hydroxide and rock again.

14. Finally, holding the syringe vertically with needle hanging downward push the plunger in until its end reaches the graduation marks and read the amount of air remaining in the syringe. The difference between this reading and the one taken in step 12 equals the amount of CO_2 extracted from 0.1 cc. of medium, best expressed as volumes or as volumes per cent; *e.g.*, 0.1 cc. of CO_2 from 0.1 cc. of medium equals 1 volume or 100 volumes per cent.

It is our experience that more accurate results are obtained with samples of 0.1 cc. of medium than with larger amounts. This is doubtless due to the fact that in such a small syringe as we use the amount of air that can be passed through the sample and held within the syringe for measurement is limited and although sufficient for complete aeration of 0.1 cc. of medium is hardly sufficient for a larger sample. Without doubt larger samples could be employed in a longer syringe. It is an advantage to color the acid and alkali solutions with indicators, methyl red in the acid and thymol blue in the alkali, so that one may be sure of the reaction of the contents of the syringe at all times.

Accuracy of the Determinations.

The determinations in Table I were made from a single culture of an anaerobic organism in 5 cc. of plain bouillon under vaseline. The results illustrate the possibility of obtaining uniform results with multiple determinations and various methods of expressing the results.

TABLE I.

Multiple Determinations of Carbon Dioxide Produced by an Anaerobic Organism in Plain Bouillon.

Initial CO₂ content of medium = 0.0

Final hydrogen ion concentration of culture = pH 6.5

Total gas above medium at 37°C. = 1.05 volumes per 1.0 volume of medium.

Determinations of CO ₂ above medium.						
Sample, cc.	0.75	0.75	0.45	0.45	0.1	0.1
CO ₂ , per cent.	41.0	40.0	40.0	39.0	40.0	39.0
H ₂	<u>59</u>	<u>60</u>	<u>60</u>	<u>61</u>	<u>60</u>	<u>61</u>
CO ₂	41	40	40	39	40	39
CO ₂ (vol. at 37°C.)	0.43	0.42	0.42	0.41	0.42	0.41

Determinations of CO ₂ in medium* (samples = 0.1 cc.).						
CO ₂ (vol. at 20°C.)	0.55	0.5	0.5	0.55	0.5	0.5

Computation of total CO ₂ .	
Average; CO ₂ above medium	0.42 vol. at 37°C.
“ CO ₂ in medium	0.52 vol. at 20°C. or <u>0.55</u> “ “ 37° “
Total CO ₂ per 1.0 vol. of medium	0.97 “ “ 37° “

* By CO₂ in medium is meant not only CO₂ present as such but also that present in the form of carbonates or carbonic acid.

To determine how much of the CO₂ present as carbonate might be recovered by the above method from water or from bouillon the following experiment was performed. 0.5066 gm. of sodium oxalate was converted into sodium carbonate by ignition in a platinum crucible. The sodium carbonate was dissolved in 10 cc. of distilled water and 3 cc. of this solution were diluted to 25 cc. with distilled water and with plain bouillon respectively for the determinations to be described.

As a control 3 cc. of distilled water were added to 22 cc. of the bouillon without addition of carbonate. Quadruple determinations were made of the CO_2 recovered from each of the three solutions. The amount of each sample taken was 0.1 cc. The results are presented in Table II.

TABLE II.

Determinations of Carbon Dioxide in Standard Solutions of Sodium Carbonate in Water and in Bouillon.

Room temperature = 20°C . Barometric pressure = 765.2 mm. Hg.

Hydrogen ion concentration of bouillon before addition of Na_2CO_3 = pH 7.3

	Determinations.				Average.
CO_2 from Na_2CO_3 in water	1.05	1.05	1.05	1.03	1.045 vol. in 1.0 vol. of solution.
CO_2 " Na_2CO_3 " bouillon	1.02	1.05	1.05	1.05	1.042 vol. in 1.0 vol. of solution.
CO_2 " H_2O " "	0.02	0.0	0.03	0.0	0.012 vol. in 1.0 vol. of solution.

Calculated theoretical volume of CO_2 in the above solutions of Na_2CO_3 in water and in bouillon = 1.066 volume in 1.0 volume of solution at 20°C . and 765.2 mm. Hg.

Experiments Illustrating the Technical Possibilities of the Methods.

Although the conditions under which a culture grows in the vaseline tube are not the same as those in the Smith fermentation tube and one would therefore not expect to obtain identical results in the two tubes, it seemed worth while to compare the results obtained by both methods with a view to interpreting their differences. A number of series of experiments have been performed with this end in view. The following is typical.

A strain of *Bacterium coli* was inoculated into six vaseline tubes and five fermentation tubes containing a certain lot of 2 per cent dextrose bouillon (pH = 7.2). By the 4th day of incubation all tubes had ceased to show changes in gas volume. The results of analysis of the gas above the media on the 4th day are recorded in Table III.

It should be noted that the total volume of gas produced above the medium in the two kinds of tubes cannot be compared because the

gas is measured in different terms. In the fermentation tube the gas collected comes from a diminishing amount of culture as gas formation forces the medium over into the open bulb and the gas is measured in terms of the capacity of the closed arm of the tube. In the vaseline tube the volume of the culture medium remains constant and the gas is measured in terms of volume of the medium. It is for this reason

TABLE III.

Gas Formation by Dextrose Bouillon Cultures of Bacterium coli in Smith Fermentation Tubes and in Vaseline Tubes.

Fermentation tubes.		Vaseline tubes.	
Gas above medium.	$\frac{H_2}{CO_2}$	Gas above medium.	$\frac{H_2}{CO_2}$
<i>per cent</i>		<i>vol.</i>	
65	$\frac{53}{47}$	0.95	$\frac{48}{52}$
41	$\frac{55}{45}$	0.9	$\frac{49}{51}$
46	$\frac{55}{45}$	0.9	$\frac{50}{50}$
49	$\frac{51}{49}$	0.9	$\frac{50}{50}$
51	$\frac{56}{44}$	0.9	$\frac{50}{50}$
		0.9	$\frac{50}{50}$

that we prefer to avoid the use of the term "per cent" in stating the volume of gas produced in the vaseline tube. It is difficult to see how one set of values can be translated into terms of the other. Aside from this fact it is noted that the results in the vaseline tubes are somewhat more uniform than those in the fermentation tubes. It is also found that invariably a larger proportion of CO_2 is formed under vaseline than in the closed arm of the fermentation tube. The reason for this is, as pointed out by Keyes (1909), that CO_2 is much more

soluble in water than is hydrogen and therefore not only does a considerable volume of CO₂ pass into solution in the medium but in the fermentation tube passes through the medium out into the air.

As a means of studying gas production the vaseline tube has many points of similarity to the long agar tube of Burri and Dügge (1909) used by Frieber (1913, *a* and *b*) for gas analyses. In this tube, however, only solid medium was used and a layer of sterile non-nutrient agar was used instead of vaseline as a seal. The technical possibilities of the vaseline tube are much greater.

TABLE IV.

Gas Formation by Dextrose Bouillon Cultures of Bacterium coli in Vaseline Tubes under Anaerobic and Aerobic Conditions.

Vaseline tubes (without air).		Vaseline tubes (plus 1.0 vol. of air).	
Gas above medium.	CO ₂	Gas above medium.	CO ₂
<i>vol.</i>	<i>per cent</i>	<i>vol.</i>	<i>per cent</i>
0.95	52	1.95	38
0.9	51	1.9	38
0.9	50	1.95	36
0.9	50	1.9	38
0.9	50	1.95	35
0.9	50	1.95	36
Average.....0.91	50.5	1.93	36.8
$0.91 \times 0.505 = 0.46 \text{ vol. of CO}_2$		$1.93 \times 0.368 = 0.71 \text{ vol. of CO}_2$	

In the methods of all the authors referred to above the cultures were grown under anaerobic conditions. Eldredge and Rogers (1914) and Osterhout (1918) have devised forms of apparatus for studying CO₂ production under aerobic or at least partially aerobic conditions. Some of the results obtained by the use of the tube of Eldredge and Rogers will be referred to later. The vaseline tube also permits the study of gas production under controlled aerobic conditions. After the medium in the tube has been inoculated and the vaseline allowed to solidify on the surface a measured volume of air or other gas may be injected beneath the vaseline plug by means of a syringe and the long needle used for gas analysis. The passage made by the needle in the vaseline is easily closed by means of a warm spatula as the needle

is withdrawn. In the experiment partially recorded in Table III was also included a series of six similar vaseline tubes of the same medium inoculated with the same strain of *Bacterium coli* but injected with an equal volume of air just after inoculation. Gas analyses were made at the same time as were those recorded in Table III. A comparison of the results with those of the vaseline tubes without air is given in Table IV.

Experiments were carried out with various volumes of air injected under the vaseline plug. With decreasing amounts of air below 1 volume the results gradually approach those obtained under anaerobic conditions. With 2 volumes of air the results were very nearly the same as with 1 volume. Naturally these proportions must be regarded as valid for this culture and medium only.

If from the 1.93 volumes of gas found in the tubes containing air the volume of air injected be subtracted, there remains 0.93 volume of gas produced by the culture, and since 0.71 volumes of CO_2 were present it might be computed that the gas ratio under aerobic conditions was $\frac{\text{H}_2}{\text{CO}_2} = \frac{22}{71}$, or $\frac{24}{76}$, as compared with $\frac{\text{H}_2}{\text{CO}_2} = \frac{50}{50}$ under anaerobic conditions. This calculation, however, assumes that the air injected is entirely inert and takes no part in the reaction. This we have reason to believe is not the case.

Determination of Oxygen in the Gas above the Medium.

The oxygen is readily determined in the same sample of gas used for the determination of CO_2 provided no atmospheric air is drawn into the syringe. This may be accomplished by having the space between the syringe plunger and the end of the needle filled with dilute acid instead of air as the needle is thrust through the vaseline plug. Otherwise the CO_2 is determined in the same manner described above and then the needle is dipped into a concentrated aqueous solution of pyrogalllic acid. A little of the pyrogalllic acid solution is drawn up into the syringe and as it mixes with the sodium hydroxide and absorbs the oxygen present more of the pyrogalllic acid is automatically sucked up. The process is slower than the absorption of CO_2 by sodium hydroxide but, with constant rocking of the syringe back and forth, is complete in 2 or 3 minutes and the reading is made.

In another experiment with the same strain of *Bacterium coli* as used for the results given in Tables III and IV, both the CO₂ and the O₂ above the medium were determined and also the CO₂ in the medium. The results are given in Table V.

The outstanding feature of this experiment was the disappearance of the oxygen from the air above the aerobic culture. Assuming that

TABLE V.

Further Gas Analysis of Dextrose Bouillon Cultures of Bacterium coli under Anaerobic and Aerobic Conditions.

	Vaseline tubes (without air).		Vaseline tubes (plus 1.15 vol. of air).	
	Culture.	Control.	Culture.	Control.
	vol.	vol.	vol.	vol.
Gas above medium.....	1.1	0.0	2.15	1.15
CO ₂ " "	0.5	0.0	0.75	0.04
CO ₂ in "	0.25	0.05	0.15	0.05
Total CO ₂	0.75	0.05	0.9	0.09
O ₂ above medium.....	0.0	0.0	0.0	0.07

Oxygen determined in atmosphere = 0.2 vol.

this was the case also in the experiment of Table IV, consideration of this factor would increase the gas ratio under aerobic conditions to $\frac{H_2}{CO_2} = \frac{37}{63}$ instead of $\frac{24}{76}$ since the space assumed to be occupied by oxygen was in reality occupied by some gas² other than CO₂ or O₂ produced by the culture. The disappearance of a part of the oxygen in the aerobic control tube noted in Table V was probably due to its passing into solution in the air-free medium which had been kept tightly sealed since it was autoclaved. The small amounts of CO₂ found in the controls may or may not be significant. It is not surprising that less CO₂ was found in the medium of the aerobic culture than in the anaerobic culture since the presence of air above the former favored the outward diffusion of CO₂. Taking all the figures into

² In stating the gas ratio $\left(\frac{H_2}{CO_2}\right)$ we have followed the custom of regarding all of the gas produced by the culture, other than CO₂, as hydrogen.

consideration it appears that *Bacterium coli* not only produces more CO₂ under aerobic conditions but a larger proportion of CO₂ with respect to H₂ or other gases.

Certain organisms which have been regarded as non-producers of gas have been found by special methods to produce appreciable amounts of CO₂. Such results are interesting and open up new possibilities for classification and for physiological study. However, they do not invalidate the practical value of formerly used methods for distinguishing between so called "gas-producing" and "non-gas-producing" organisms under stated conditions of cultivation. It was shown by Hesse (1893) that *Bacterium typhosus* and many other bacteria ordinarily considered to be non-gas producers do produce an appreciable amount of CO₂ and consume O₂ in the process. He calls this process the respiratory activity of bacteria.

Ayers, Rupp, and Mudge (1921) used the tube of Eldredge and Rogers (1914) to demonstrate CO₂ production by streptococci, and Nichols (1921) demonstrated CO₂ production by *Bacterium typhosus* by the same means. In this tube cultures are grown under aerobic conditions. Since, however, the atmosphere of the tube above the medium is kept free of CO₂ by the barium hydroxide solution we would expect to find less CO₂ in the medium than when the aerobic vaseline tube is used. To compare the results obtained in the vaseline tube with those of the Eldredge tube we must therefore consider the CO₂ in the medium as well as that above it in the vaseline tube. We have done this with two strains of streptococci for which we are indebted to Dr. Ayers. Strain X-4 was reported by Ayers, Rupp, and Mudge as producing CO₂ from dextrose and Strain 16H-1 was reported to produce CO₂ from Bacto-peptone. In Table VI are given the total CO₂ determinations of Ayers' strains of streptococci in various media. The determinations were made after incubation at 37°C. for 6 days. The amount of medium used was 3 cc. in each tube.

The resemblance between the CO₂ determinations of our aerobic cultures and those of Ayers, Rupp, and Mudge obtained by an entirely different method is so close that the differences seem insignificant. It is to be noted, however, that under anaerobic conditions much less CO₂ is produced in all media. Possibly this explains failure to demonstrate gas production by streptococci under the anaerobic conditions imposed by other methods.

Using the tube of Eldredge and Rogers, Nichols (1921) demonstrated CO₂ production by *Bacterium typhosus*. He obtained the maximum amount of CO₂ from cultures in 1 per cent glucose extract broth. In 2 per cent glucose veal infusion broth and with a different strain of typhoid bacillus, however, we have obtained even larger amounts of CO₂. The results of our determinations are recorded in Table VII.

TABLE VI.

Determination of Total Carbon Dioxide Produced by Streptococci in Vaseline Tubes under Anaerobic and Aerobic Conditions.

The medium was veal infusion bouillon containing 1 per cent Fairchild's peptone plus the substances indicated above each column.

Strain.	Vaseline tubes (without air).			Vaseline tubes (plus 1.0 vol. of air).		
	3 per cent Fairchild's peptone.	3 per cent Bacto- peptone.	2 per cent dextrose.	3 per cent Fairchild's peptone.	3 per cent Bacto- peptone.	2 per cent dextrose.
	vol.	vol.	vol.	vol.	vol.	vol.
Streptococcus X-4.....	0.05	0.05	0.67	0.13	0.06	1.17 (1.25)
“ 16H-1.....	0.05	0.13	0.0	0.21 (0.12)*	0.22 (0.33)	0.13 (0.09)

* The bold faced figures in parentheses are the results of Ayers, Rupp, and Mudge reduced to the same terms as ours. It should be noted, however, that whereas one of their media contained 4 per cent of Bacto-peptone ours used for comparison contained 3 per cent of Bacto-peptone and 1 per cent of Fairchild's peptone.

There was good growth of the typhoid bacillus under anaerobic conditions, though somewhat better under aerobic conditions. It is again seen that a really significant amount of CO₂ is produced under aerobic conditions only, and that a part but not all of the oxygen was consumed.

It must be pointed out that in interpreting the results of the determination of CO₂ in and above the medium certain very important factors must be taken into consideration. There is a very intimate relationship between temperature, hydrogen ion concentration of the culture, and the proportionate amount of CO₂ in and above the me-

dium. For instance, if the culture becomes alkaline, as may be the case with certain anaerobes, a large proportion of the CO_2 will be found in the medium as carbonates. If it becomes strongly acid a large proportion of the CO_2 will be found in the gas above the medium. If the culture is transferred from the incubator to the refrigerator the solubility of gases in the medium is appreciably increased. If the vaseline plug is broken so that atmospheric air gains access to the culture, CO_2 begins to pass out of the medium into the air. The latter can be demonstrated easily by breaking the seal of a culture and making periodic determinations of the CO_2 in the medium.

TABLE VII.

Determination of Carbon Dioxide Produced by Bacterium typhosus in Dextrose Bouillon under Anaerobic and Aerobic Conditions.

	Vaseline tube (without air).	Vaseline tubes (plus 1.0 vol. of air).	
	Culture.	Culture.	Control.
	vol.	vol.	vol.
Gas above medium.....	0.0	1.1	1.0
CO_2 " "	0.0	0.14	0.03
CO_2 in "	0.1	0.15	
Total CO_2	0.1	0.29	
O_2 above medium.....	0.0	0.06	0.13

Oxygen determined in atmosphere = 0.2 vol.

Sterilization, Inoculation, and Cleaning of Vaseline Tubes.

Before summarizing the advantages of the technique described it may be well to describe one or two simple points of technique which serve to make the vaseline tube almost as convenient to handle as an ordinary test-tube.

Autoclaving cannot be relied upon to sterilize vaseline since it is essentially a "dry" substance which the steam does not penetrate. It may be perfectly sterilized without visible alteration along with glassware in the hot air sterilizer at $175\text{--}185^\circ\text{C}$. for 2 hours. After being so sterilized it may be pipetted onto non-sterile medium in the tubes and then autoclaved with the medium. During sterilization air is driven from the medium and if the tubes are promptly cooled

after coming from the autoclave the medium under the solid vaseline plug is preserved free from air for long periods of time. There is no danger of spilling if tubes are turned over, and the medium may be kept at room or incubator temperature without evaporation. Since there is no water vapor in the tubes above the vaseline there is no tendency for molds to grow through the cotton plugs.

It is not necessary to pass a pipette through the vaseline to inoculate or add anything to the medium in the tube. In fact it may be inoculated with a platinum loop if the vaseline seal is opened as shown in Figs. 4 and 5. The vaseline is melted by directing the flame of a micro burner against the side of the tube. The tube is then slanted in a dish or tray of water as shown in Fig. 4. After the vaseline has hardened as a layer over the slanted surface of the medium the tube is rotated and gently tapped by the fingers, as shown in Fig. 5, until the vaseline over the medium flaps up and adheres to the side of the tube, exposing the medium for inoculation. The seal is again closed by melting the vaseline by means of the flame directed against the outside of the tube in the region of the vaseline. The medium need not be perceptibly heated by the process and with reasonable care tubes rarely crack.

Tubes of discarded cultures are cleaned as follows: The tubes are placed upright in a basket and autoclaved. While they are still hot the cotton plugs are withdrawn, the basket is placed in a deep pail or other vessel somewhat deeper than the tubes, and the tubes, remaining upright, are filled and covered with hot water. The pail of water is heated on a burner while the vaseline rises to the surface whence after cooling it may be recovered if desired. The tubes may then be washed by the usual method.

SUMMARY.

There has been described the use of the vaseline tube and the tuberculin syringe for the study of gas production by bacteria.

A comparison is made of some of the results obtained by the use of the method here described, the Smith fermentation tube, and the tube of Eldredge and Rogers.

The reports of CO₂ production by certain streptococci by Ayers, Rupp, and Mudge and by *Bacterium typhosus* by Nichols have been confirmed by the author's method.

The data presented serve to illustrate the accuracy and technical possibilities of the method.

In addition to economy of glassware, medium, and labor, the vaseline tube and syringe method of micro gas analysis possesses the following advantages. (1) Gas produced above either liquid or solid media may be measured and analyzed. (2) The gas produced may be measured in terms of a definite and constant quantity of medium used. (3) The vaseline tube provides a closed system from which gases do not escape into the air. (4) Separate determinations of the CO₂ produced in and above fluid media may be made. (5) Determinations may be made from very small samples of material. (6) Numerous gas analyses of the same culture may be made at various times during the growth of the culture without contaminating or destroying it. (7) Gas production may be observed under both anaerobic and controlled aerobic conditions.

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EXPLANATION OF PLATES.

PLATE 55.

FIG. 1. Withdrawing the sample of gas from beneath the vaseline plug.

PLATE 56.

FIG. 2. Sodium hydroxide solution being drawn into the syringe for the absorption of carbon dioxide.

FIG. 3. The aeration of a sample of culture for the determination of the carbon dioxide in the medium.

PLATE 57.

FIG. 4. The tube of medium slanted but still covered by a layer of vaseline.

FIG. 5. The slanted tube rotated causing the lower end of the vaseline seal to flap up, thus exposing the medium for inoculation.



FIG. 1.

(Brown: Micro gas analysis of bacterial cultures.)

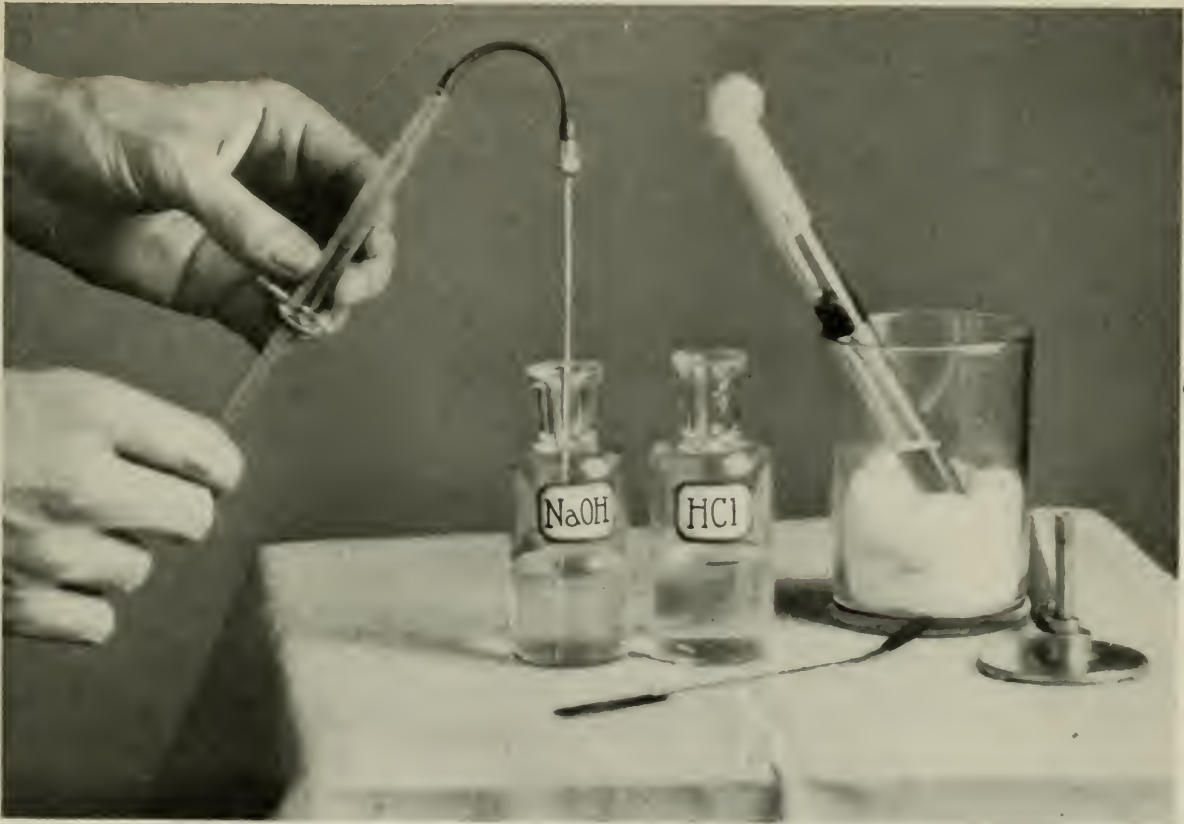


FIG. 2.



FIG. 3.

(Brown: Micro gas analysis of bacterial cultures.)

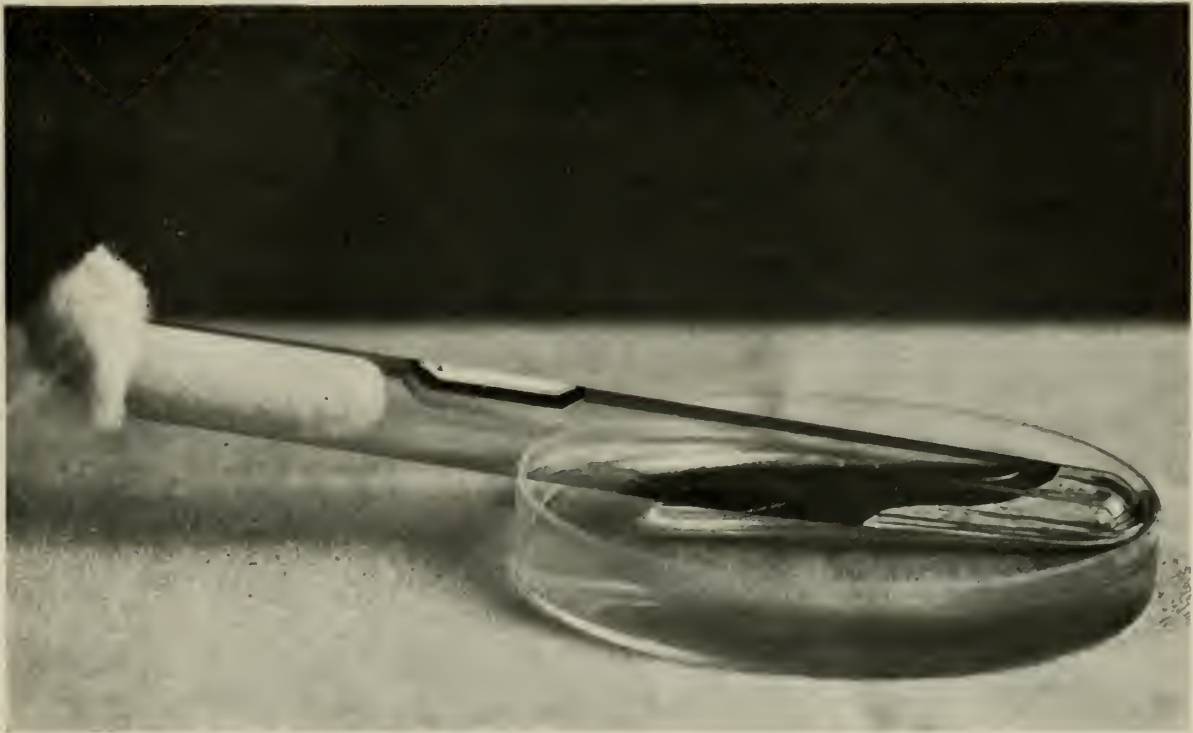


FIG. 4.



FIG. 5.

(Brown: Micro gas analysis of bacterial cultures.)

STUDIES ON THE PNEUMOCOCCUS.

I. ACID DEATH-POINT OF THE PNEUMOCOCCUS.

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(Received for publication, December 16, 1921.)

In a previous publication¹ we brought forward evidence indicating that in the growth and death of the pneumococcus in fluid cultures containing 1 per cent glucose the production of acid was the most important bactericidal factor and that such cultures when allowed to grow and die out usually reach a final pH of about 5.1. In our experiments on the relation of the time of exposure to the death of the organisms we made use of weak solutions of bouillon at varying hydrogen ion concentrations. In order to exclude the possibility that an unsuitable medium might play a part in these results, we have since used a stronger broth adjusted to approximate isotonicity, as shown in Table I.

5 cc. from each flask (Table I) were removed to sterile test-tubes and each tube of the series was inoculated with 2 drops of a saline suspension of washed living pneumococci. Transplants were made on blood serum after varying periods of incubation. Table II indicates the results obtained in an experiment with Type I pneumococcus. No noteworthy change in the hydrogen ion concentration of the tubes occurs during the experiment. It will be seen that at a pH of 4.47 no living organisms were obtained after an interval of 1 hour. At a pH of 5.15 living organisms were obtained after 1 hour but not after 2. At a pH of 5.78 organisms were living after 2 hours but not after 3, while at a pH of 6.33 organisms were obtained after 6 hours. At a pH of 6.63 to 7.80 inclusive transplants were positive from 8 to 10 days.

A considerable number of similar experiments has been performed

¹ Lord, F. T., and Nye, R. N., *J. Exp. Med.*, 1919, xxx, 389.

TABLE I.
Isotonic Broth for Testing the Acid Death-Point of the Pneumococcus.

125 cc. of double strength bouillon (broth made with double strength beef infusion) hydrochloric acid, 0.5 M phosphates, and water to 237.5 cc.								190 cc. from each flask and sodium chloride solution to depress freezing point, plus water to approximately 200 cc.			
Flask No.	pH*	0.5 M KH ₂ PO ₄	Na ₂ HPO ₄	HCl		NaOH (concentrated).	Depression of freezing point.	10 per cent NaCl.	H ₂ O	Final depression of freezing point.	Final pH.†
				$\frac{N}{I}$	Concentrated.						
		cc.	cc.	cc.	cc.	cc.	°C.	cc.	cc.	°C.	
1	4.9	15.0		1	0.3		0.450	6.7	3.3	0.620	4.47‡
2	5.3	15.0		1	0.2		0.378	7.4	2.6	0.622	5.15
3	5.75	15.0		1	0.1		0.390	8.0	2.0	0.584	5.78
4	6.1	15.0					0.322	11.4		0.638	6.33
5	6.4	13.8	1.2			0.3	0.378	8.4	1.6	0.598	6.63
6	6.9	9.45	5.55			0.3	0.349	9.5	0.5	0.571	7.01
7	7.5		15.00				0.328	11.0		0.700	7.51
8	7.8	1.2	13.8			0.1	0.305	11.0		0.621	7.80

* Determined colorimetrically.
† Determined electrometrically.
‡ The figure in the hundreds place is uncertain by this method.

TABLE II.
Acid Death-Point of the Pneumococcus. Time Relation at Varying Hydrogen Ion Concentrations.

Flask No.	pH*	Results obtained on transplants after varying intervals.																	Final pH.†		
		1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	24 hrs.	48 hrs.	72 hrs.	4 days.	5 days.	6 days.	7 days.	8 days.	9 days.	10 days.		12 days.	13 days.
1	4.47	0‡	0																	4.47	
2	5.15	+	0	0																5.15	
3	5.78	+	+	0	0															5.72	
4	6.33	+	+	+	+	+	+	0	0											6.19	
5	6.63	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	0	0		6.58	
6	7.01	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	0	0		6.58	
7	7.51	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	0	0		7.14	
8	7.80	+	+	+	+	+	+	+	+	+	0	+	+	+	+	0	+	+	0	0	7.30

* Determined electrometrically.
† Determined colorimetrically.
‡ 0 indicates lack of growth on the transplant; +, growth.

with Type I and II pneumococci. Though there is slight variation in the time element in the bactericidal action of acidity, the results are essentially the same for the two types and confirm the results previously obtained and reported; at a pH of about 5.1 or lower the pneumococcus does not survive longer than a few hours, at a pH of about 6.8 to 7.4 the pneumococcus may live for at least many days, and in the intervening solutions, between 6.8 and 5.1, the organism is usually killed with a rapidity which bears a direct relation to the hydrogen ion concentration; *i.e.*, the greater the acidity the more rapid the death.

For technical assistance in the experiments in this and the subsequent articles in this series we are indebted to Miss Ruth Seybolt and Miss Elnora Reed Blanchard.

STUDIES ON THE PNEUMOCOCCUS.

II. DISSOLUTION OF PNEUMOCOCCI AT VARYING HYDROGEN ION CONCENTRATIONS. EFFECT OF TEMPERATURE, PREVIOUS KILLING OF THE ORGANISMS, AND FRESH HUMAN SERUM ON THE PHENOMENON. BEHAVIOR OF OTHER ORGANISMS.

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(Received for publication, December 16, 1921.)

In a previous publication¹ we have called attention to the dissolution after incubation of living washed pneumococci, suspended in standard solutions of known hydrogen ion concentration. There is clearing of the suspensions from disintegration of the organisms within the range of pH of about 5.0 to 6.0 within an hour, and diminishing density with longer incubation in the more alkaline side of the scale. In discussing the phenomenon, we suggested that an enzyme derived from the organisms might be responsible for their dissolution. Further experiments have shown that the phenomenon is constantly observed with all the strains of pneumococci tested, including examples of the types commonly known as Types I, II, and III.

Avery and Cullen² have since demonstrated the presence in the pneumococcus of an erepsin-like enzyme, a lipase, an invertase, an amylase, and an inulase, with an activity within a zone of hydrogen ion concentration which bears a striking correlation to that of the biologic activity of the bacterial cell.

To obtain further information on the dissolution phenomenon, it seemed desirable (1) to suspend the organisms in another solution than the standard, (2) to determine the effect of temperature on the phenomenon, (3) to observe the behavior of dead as well as living pneumococci, (4) to test the effect of fresh human serum on the dissolution of pneumococci, (5) to compare the rate of dissolution of pneumococci

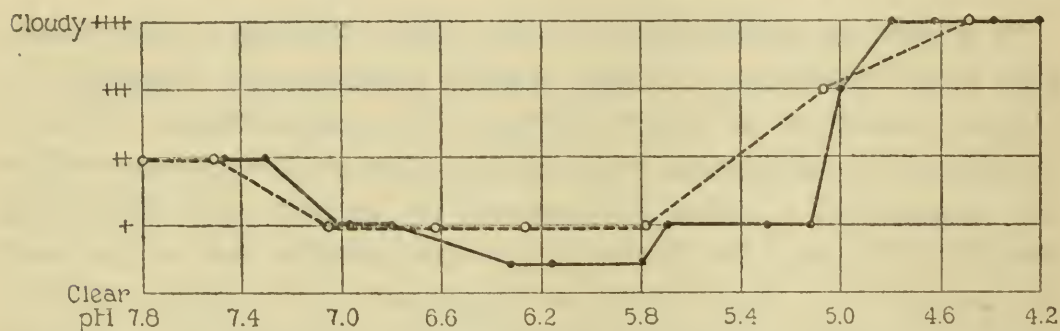
¹ Lord, F. T., and Nye, R. N., *J. Exp. Med.*, 1919, xxx, 389.

² Avery, O. T., and Cullen, G. E., *J. Am. Med. Assn.*, 1920, lxxiv, 1668; *J. Exp. Med.*, 1920, xxxii, 547, 571, 583.

in a fresh standard solution at pH 6.1 with that in a standard solution of the same pH in which the organisms had previously undergone dissolution, and (6) to do comparative dissolution tests on pneumococci and other organisms.

1. *Dissolution of Pneumococci in Nutrient Broth at Varying Hydrogen Ion Concentrations. Comparison with Dissolution in Standard Solutions.*—To observe the effect of suspending pneumococci in a medium in which the conditions are suitable for growth and multiplication at the proper range of pH, a double strength meat infusion broth was made and the pH and tonicity were adjusted as shown in the preceding article.³

To each of eight tubes containing 0.2 cc. of approximately isotonic ($\Delta = 0.580 - 0.748^\circ\text{C}.$) standard solution at varying hydrogen ion

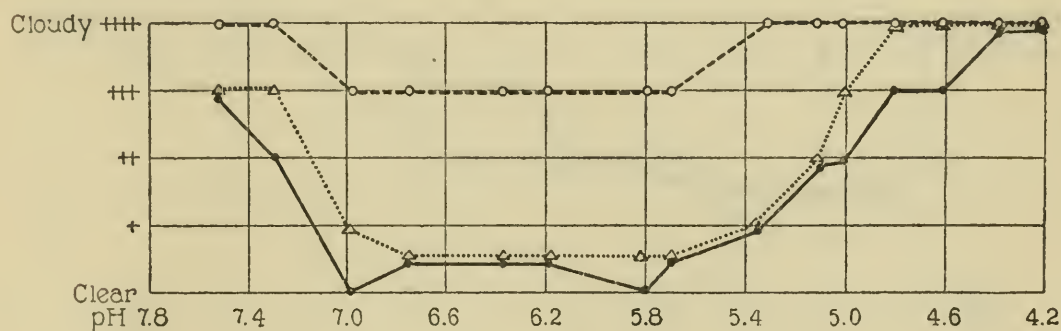


TEXT-FIG. 1. Dissolution of pneumococci in nutrient broth and standard solutions at varying hydrogen ion concentrations. Comparison of the dissolution of *Pneumococcus* Type I at the end of 24 hours at varying hydrogen ion concentrations in isotonic bouillon (broken line) and in isotonic standard solutions (solid line).

concentrations and to each of a similar number of tubes containing an equal amount of approximately isotonic nutrient broth at varying hydrogen ion concentrations, 0.2 cc. of a saline suspension of living washed *Pneumococci* Type I was added. The two series were incubated. No change in the suspensions was observed at the end of 1 hour. At the end of 6 hours there was slight dissolution in both series at about the same range of pH; *i.e.*, at 5.78 to 7.8 in the bouillon series and 5.14 to 6.98 in the standard solutions. The result at the end of 24 hours is shown in Text-fig. 1. The dissolution in the two series was practically the same and almost complete at a pH of about 5.7 to 7.0.

³ Lord, F. T., and Nye, R. N., *J. Exp. Med.*, 1922, xxxv, 686, Table I.

2. *Effect of Temperature on Dissolution of Pneumococci at Varying Hydrogen Ion Concentrations.*—Living washed Pneumococci Type I were suspended in approximately isotonic standard pH solutions at incubator and room temperature (23.2°C.) and in the ice box (12.2°C.). The series used for the test in the ice box was placed on ice and when thoroughly chilled a chilled suspension of organisms was added. The tubes were then replaced on ice. Observations were made after 1, 6, and 24 hours. No change in the density of the suspensions was noted after 1 hour. After 6 hours no change was observed in the suspensions at room and ice box temperature, but in the suspensions at incubator temperature there was slight clearing at pH 4.81 and 5.02, more marked clearing at pH 5.14 to 6.98, and slight clearing at pH 7.3 to 7.49. The appearance of the tubes after 24 hours is indicated in

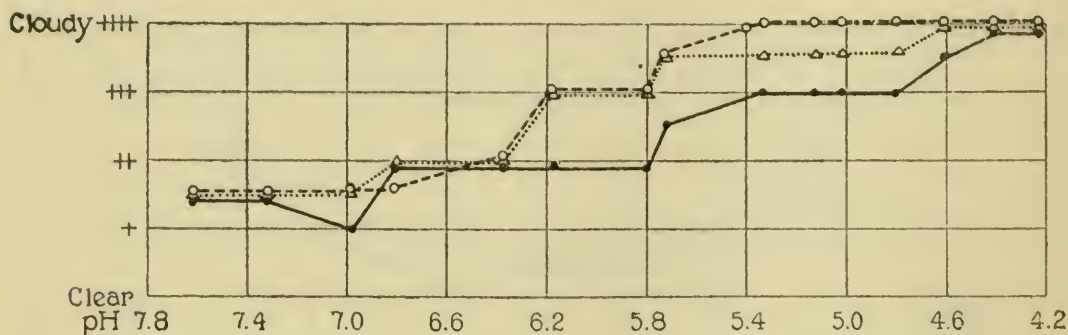


TEXT-FIG. 2. Effect of temperature on the dissolution of pneumococci. Comparison of the degree of dissolution of living *Pneumococcus* Type I suspended in isotonic standard pH solutions in the incubator (solid line), at room temperature (dotted line), and in the ice box (broken line). The observations were made after 24 hours.

Text-fig. 2. At the expiration of this interval dissolution had proceeded to about the same degree at incubator and room temperature and was almost complete at pH 5.33 to 6.98. Though dissolution occurs after 24 hours at ice box temperature and at the same range of pH, it is much less marked than at incubator and room temperature. After the expiration of 48 hours, however, the suspensions at ice box temperature had reached the same degree of dissolution as those at incubator and room temperature from a pH of 5.8 to 6.98 inclusive. Dissolution in the ice box was somewhat less at pH 5.02 to 5.72 inclusive.

Dissolution therefore takes place at room and ice box temperature, though much more slowly than at incubator temperature. It may be noted in this as in the preceding experiment that dissolution occurs more quickly at a certain critical range; *i.e.*, from about pH 5.14 to 6.98.

3. *Effect of Previous Death of Pneumococci on the Dissolution Phenomenon.* (a) *Dissolution of Pneumococci Allowed to Grow and Die Out.*—A glucose broth culture of pneumococci was allowed to grow and die out. At the end of about 72 hours the pH was 5.02 and a transplant was sterile. The culture was centrifuged, the sediment suspended in isotonic ($\Delta = 0.62^\circ\text{C}.$) saline solution, again centrifuged, and resuspended in isotonic saline solution. An equal amount (0.2 cc.) of suspended organisms was added to three sets of approxi-

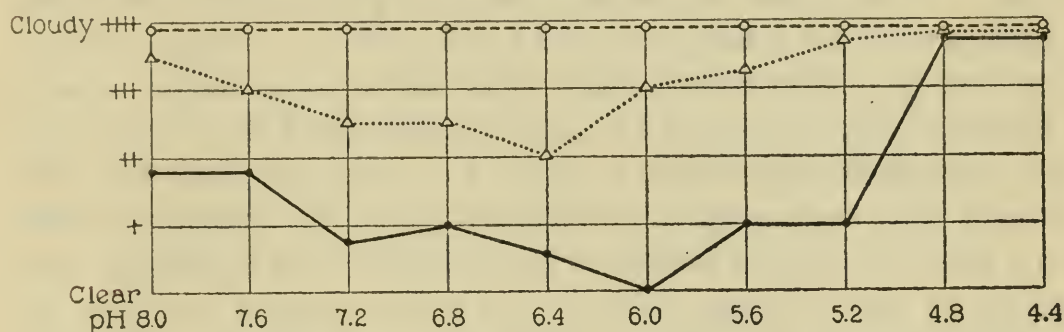


TEXT-FIG. 3. Effect of previous death of pneumococci on dissolution. Comparison of the degree of dissolution of *Pneumococcus* Type I allowed to grow and die out in glucose broth, suspended in isotonic standard solutions, at incubator temperature (solid line), at room temperature (dotted line), and in the ice box (broken line). The observations were made after 24 hours.

mately isotonic standard pH solutions, ranging from 7.49 to 4.23. One series was placed in the incubator, another in the ice box ($11.2^\circ\text{C}.$), and the third left at room temperature ($20.0^\circ\text{C}.$). Observations were made after 1, 18, and 24 hours. Dissolution slowly proceeded to about the same degree in all three sets and at about the same range of pH. The results are shown in Text-fig. 3. The degree of dissolution of dead organisms increases progressively from the acid toward the alkaline end of the scale and is in general most complete at a pH of 7.0 to 7.49. On comparison of the dissolution of living (Text-fig. 2) and dead organisms (Text-fig. 3) a difference is seen in the curves. After 24 hours the dissolution of living organisms is most marked and

almost complete within a range of pH from 5.72 to 6.98, and takes place little or not at all at the more alkaline and more acid ends of the scale respectively. Death of pneumococci allowed to grow and die out may be ascribed to acid production in the media, and the dissolution curve of organisms thus killed is different from that of organisms killed by heat (*cf.* Text-fig. 4).

(b) *Dissolution of Pneumococci Killed by Heat.*—To determine the effect of previous killing of the pneumococci by heat, one lot of organisms at the height of their growth in dextrose bouillon was heated to 100°C. for 5 minutes, a second lot was heated to 57°C. for 1 hour, and a third lot of living organisms was reserved as a control. Transplants from the flasks heated at 100°C. for 5 minutes and 57°C. for 1 hour were sterile. All three lots were centrifuged, washed with



TEXT-FIG. 4. Effect of heating pneumococci on dissolution. Comparison of the degree of dissolution of *Pneumococcus* Type II, living (solid line), killed by heat at 57°C. for 1 hour (dotted line), and killed by heat at 100°C. for 5 minutes (broken line). The observations were made after 24 hours in the incubator.

sterile isotonic saline solution, and recentrifuged. The supernatant saline solution was pipetted off and a cloudy suspension of the organisms made by adding fresh sterile isotonic saline solution, care being taken to secure suspensions of equal density in the three groups. The three lots were then mixed with standard pH solutions, placed in the incubator, and the results noted at intervals. As shown in Text-fig. 4, after 24 hours the suspensions of living pneumococci showed the usual dissolution curve, the clearing of the suspension being most marked at a pH of 6.0. No dissolution was noted at a pH of 4.8 and 4.4. Considerable, though less dissolution than at a pH of 6.0 was observed in the intervening acid end of the scale and a gradually dimin-

ishing clearing of the tubes from a pH of 6.0 toward the more alkaline end of the scale. Some dissolution of pneumococci killed by heating at 57°C. for 1 hour was observed, the general character of the curve being similar to that with the living organisms. Previous heating of the organisms at this temperature had evidently diminished their susceptibility to dissolution at the critical hydrogen ion concentrations. The pneumococci heated to 100°C. for 5 minutes showed no change in the density of the different tubes.

4. *Effect of Fresh Human Serum on Dissolution of Pneumococci at Varying Hydrogen Ion Concentrations.*—In view of the well established presence of antienzyme in normal serum it seemed desirable to determine its effect on the dissolution of pneumococci. For this purpose 0.5 cc. of fresh human serum was placed in each of seven sterile test-tubes. To each tube was added 2 drops of buffer solution containing equal parts of 0.5 M KH_2PO_4 and 0.5 M Na_2HPO_4 . 2 drops of indicator were also added to each tube, 1 per cent aqueous sodium alizarin sulfonate being used at pH 3.6 to 5.6 inclusive and 0.01 per cent aqueous phenolsulfonephthalein at pH 6.1 to 7.8 inclusive.⁴ The fluid in each tube was brought to the desired pH by the addition of drops of N HCl, 0.2 N HCl, N NaOH, or 0.2 N NaOH. The number of drops added to each tube was recorded and those receiving less than the maximum number (8 drops) were made up to volume with sterile saline solution. The comparator rack method was used in the construction of the series. Sterile precautions were used throughout and cultures from the completed series were negative.

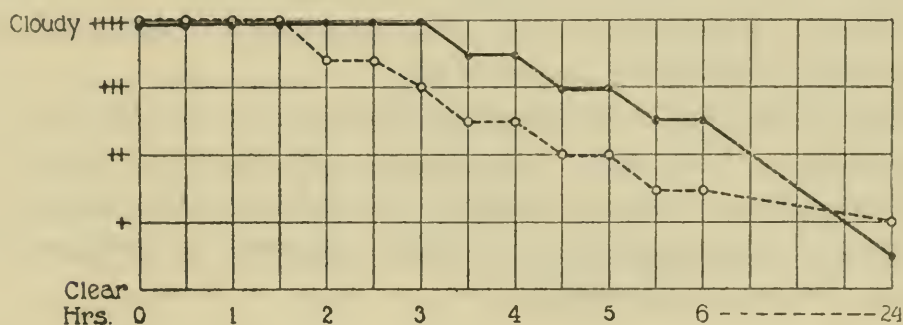
To each tube in the two series 0.2 cc. of a heavy suspension of washed living Pneumococci Type I was added. The two sets were incubated for 36 hours. The pH did not change during this interval. Macroscopic changes in density were difficult of appreciation and reliance was placed on microscopic examination of stained smears. After 36 hours, examination of stained smears from the tubes containing organisms suspended in serum at varying pH showed no apparent dissolution in any of the tubes. The organisms suspended in standard pH solutions showed the usual curve of dissolution.

Suspension of pneumococci in normal serum at varying hydrogen ion

⁴ Experiments have shown that these two indicators themselves do not prevent dissolution and the inhibition is to be ascribed to the serum.

concentrations therefore prevents the dissolution of the organisms at the critical range of pH.

5. *Comparison of the Degree of Dissolution of Pneumococci in Standard Solution of pH 6.19 with Dissolution in the Supernatant Fluid of the Standard Solution of the Same pH in Which Large Numbers of Pneumococci Have Been Previously Dissolved.*—The most likely explanation of the dissolution of pneumococci at the critical range of pH in standard solutions seems to be the activation of an intracellular enzyme. If the enzyme is set free by the process of dissolution and exists in the fluid in which the organisms are undergoing disintegration, then dissolution of a fresh lot of pneumococci should take place at a more rapid rate in fluid in which dissolution has already taken place than



TEXT-FIG. 5. Comparison of the rapidity of dissolution of *Pneumococcus* Type I at pH 6.19 in the supernatant fluid of standard solution in which pneumococci have been previously dissolved (broken line), and in fresh standard solution (solid line).

in fresh standard solutions at the same pH. For the purpose of testing this hypothesis the following experiment was performed.

The contents of a liter flask of glucose bouillon culture of living *Pneumococci* Type I were centrifuged. The sediment was collected, washed in sterile isotonic ($\Delta = 0.620^\circ\text{C.}$) saline solution, recentrifuged, and suspended in 2 cc. of isotonic salt solution. This suspension of living pneumococci was added to 5 cc. of isotonic standard solution of pH 6.15 and incubated for 48 hours. At the end of this time the suspension was centrifuged. In one test-tube was placed a part of the supernatant fluid thus obtained and in another an equal amount of fresh standard solution of pH 6.19. Equal amounts of a heavy suspension of living *Pneumococci* Type I were now added to both tubes and the result

was noted after incubation⁵ (Text-fig. 5). At the expiration of $\frac{1}{2}$ hour no difference was observed in the density of the two suspensions. After 2 hours the suspension of organisms in the supernatant fluid had cleared to a perceptibly greater degree than that in the fresh standard solution. Further observation over a period of 6 hours continued to show greater dissolution in the supernatant fluid. At the expiration of 24 hours both tubes had cleared, a result to be expected in suspensions of pneumococci at pH 6.19 after a sufficient interval. Observations were made by gross and microscopic examination. The pH did not change during the experiment. Repeated experiments showed the same result.

The theory was therefore confirmed that dissolution of pneumococci takes place more rapidly in standard solution of pH 6.19 in which large numbers of organisms have been previously dissolved than in fresh standard solution at the same pH.

6. *Dissolution Tests with Other Organisms at Varying Hydrogen Ion Concentrations.*—Tests with *Streptococcus viridans* and *haemolyticus* and *Staphylococcus aureus*, similarly performed with washed suspensions of living organisms in standard solutions at incubator temperature, showed no dissolution.

DISCUSSION.

The phenomenon of dissolution of pneumococci at critical hydrogen ion concentrations seems to bear a definite relation to the living organisms. It is most marked when living organisms are used in the experiment and takes place most quickly at a range of hydrogen ion concentrations which is slightly less acid (pH 5.5 to 6.7) than the limiting hydrogen ion concentration (pH 5.1) of growing glucose bouillon cultures and slightly more acid than the most acid reaction (pH 6.8) at which growth of organisms can be continued. It occurs, however, with the lapse of time throughout the whole range of hydrogen ion concentrations (pH 6.8 to 7.8) at which the pneumococci are biologically active.

⁵ Both at the beginning and end of the experiment the suspensions of pneumococci in the supernatant fluid are somewhat more cloudy than the suspensions in fresh standard solution because of the presence of fine particles of pneumococci not removed by the centrifuge.

As we have already noted in our previous paper, the most probable explanation seems to be the activation of an enzyme derived from the bacteria themselves. Avery and Cullen's² demonstration of the presence of enzyme in bile and in phosphate solutions of pH 6.2 in which pneumococci have undergone disintegration lends support to this point of view. The fact that the phenomenon is most marked with living organisms at incubator temperature, the inhibiting effect of death of the organisms (Text-figs. 3 and 4) and of the addition of fresh human serum to the solutions, and the greater rapidity with which dissolution takes place in standard solutions in which pneumococci have been previously dissolved (Text-fig. 5) are consistent with enzymatic action. If merely the effect of acidity on ferment action is considered, the absence of dissolution at acidities greater than about pH 4.6 coincides closely with the failure of Avery and Cullen to demonstrate any appreciable ferment activity at acidities greater than pH 4.5. The less complete dissolution and change in the character of the curve of dissolution of pneumococci allowed to grow and die out in glucose bouillon (Text-fig. 3) may be due to partial destruction of the enzyme or a change in the physical state of the bacterial cell in consequence of exposure to this acidity (pH 5.02). Avery and Cullen have shown that the activity of the peptonase and lipase obtained from the pneumococcus is not appreciably diminished by exposure to pH 5.0 for 2 hours. It may be, however, at this hydrogen ion concentration, which corresponds closely with the isoelectric points and coincident precipitation optima of various biologically important proteins, that some change has occurred to diminish the permeability of the cell membrane or to change the physical state of the bacterial cell protoplasm. Less dissolution when the pneumococci are killed by heat and the absence of dissolution after heating to 100°C. may be ascribed to partial and complete destruction respectively of the enzyme at these temperatures.

Assuming that dissolution occurs in consequence of the death of the organisms and the activities of an endocellular enzyme, the disintegration may be conceived to take place most actively at that point in the scale where the largest number of organisms are most rapidly killed with, at the same time, a minimum of injury to the cell membrane which still remains permeable for the hydrogen ions and permits the activation of the endobacterial ferment.

Enzymatic action at ice box temperature is at variance with the usual temperature range of ferment action. Dissolution proceeded much more slowly at this temperature but was finally as complete as at incubator temperature.

CONCLUSIONS.

Suspensions of living pneumococci in approximately isotonic standard solutions and in approximately isotonic bouillon with pH varying from about 4.0 to 8.0 after incubation show dissolution of organisms in those solutions having a pH higher than about 5.0. Dissolution is most marked at a critical range of about pH 5.0 to 7.0. Some dissolution also takes place toward the more alkaline end of the scale. No dissolution occurs at the most acid end of the scale.

Dissolution in the standard solutions occurs at incubator, room, and ice box temperature. It is less marked at ice box temperature. Dissolution takes place in standard pH solutions with pneumococci allowed to grow and die out in glucose bouillon but unlike dissolution with living organisms is progressive from the acid toward the alkaline end of the scale. Pneumococci killed by heat for 1 hour undergo less dissolution than living organisms, the general character of the curve being similar to that with living organisms. Pneumococci killed by heat at 100°C. for 5 minutes do not undergo dissolution. The addition of fresh human serum to the suspensions of pneumococci at varying pH prevents dissolution. Dissolution of pneumococci takes place more rapidly at pH 6.1 in standard solutions in which large numbers of pneumococci have been previously dissolved than in fresh standard solutions at the same pH.

The dissolution of pneumococci under the conditions of the experiments may be ascribed to an enzyme derived from the bacteria themselves.

Other organisms such as *Streptococcus viridans* and *hemolyticus* and *Staphylococcus aureus* do not undergo dissolution under conditions similar to those to which the pneumococcus was exposed.

STUDIES ON THE PNEUMOCOCCUS.

III. DISSOLUTION OF PNEUMOCOCCI IN PNEUMONIC CELLULAR MATERIAL AT VARYING HYDROGEN ION CONCENTRATIONS. RESISTANCE OF CERTAIN OTHER ORGANISMS TO DISSOLUTION.

BY FREDERICK T. LORD, M.D., AND ROBERT N. NYE, M.D.

(*From the Research Laboratory of the Massachusetts General Hospital, Boston.*)

(Received for publication, December 16, 1921.)

To determine the effect at varying hydrogen ion concentrations of cellular material obtained from the pneumonic lung on pneumococci and other organisms, the following experiments were performed.

The mash from a pneumonic lung due to Type I pneumococcus and preserved with chloroform and toluene was ground in a sterile mortar with some of the saline solution with which the material was originally put up. The resulting cellular material was allowed to stand on ice until the supernatant fluid was clear. The supernatant fluid and sediment were put on Löffler's blood serum. Both produced proteolysis, the cells more than the supernatant fluid. The pH of this fluid was 5.5.

The pH of the cellular material was changed by adding to 10 cc. 9 drops of normal NaOH, which gave a pH of 6.95. To another 10 cc. lot 1 drop of concentrated HCl was added, with a pH of 4.5 as the result.

Dissolution Experiment with Pneumococci.—The cellular material of varying hydrogen ion concentrations thus prepared was set up with suspensions of Type I, II, and III pneumococci as shown in Table I. As will be seen from the table the behavior in the cellular suspension at varying hydrogen concentrations was practically the same for the three types. All three types at a pH of 4.5, after incubation over night, for the most part retain their morphology. Type I pneumococcus at this pH remained Gram-positive while Types II and III became Gram-negative. All three types underwent dissolution at a pH of 5.5 and 6.95.

The dissolution of pneumococci taking place in cellular material from the pneumonic lung at a pH of 6.95 and 5.5 may be ascribed to the action of an enzyme operative at this pH. Previous experiments indicate that the enzyme may be derived from the bacteria themselves, since similar dissolution of the organisms at this pH is noted in stand-

TABLE I.

Relation of Hydrogen Ion Concentration to Dissolution of Pneumococci in Cellular Material from the Pneumonic Lung.

Tube No.	Amount of cellular suspension.			Amount of pneumococcus suspension.			Smears.	
	pH 4.5	pH 5.5	pH 6.95	Type I.	Type II.	Type III.	Made at once.	Made after incubation over night.
	<i>gtt.</i>	<i>gtt.</i>	<i>gtt.</i>	<i>gtt.</i>	<i>gtt.</i>	<i>gtt.</i>		
1	5			5			Organisms numerous, in good condition, and all Gram-positive.	Organisms well preserved, in good condition, and Gram-positive.
2		5		5				Organisms have disintegrated and disappeared.
3			5	5				Organisms have disintegrated and disappeared.
4	5				5			Organisms well preserved but all Gram-negative.
5		5			5			Organisms have disintegrated and disappeared.
6			5		5			Organisms have disintegrated and disappeared.
7	5					5		Organisms mostly well preserved, a few slightly eroded; all Gram-negative.
8		5				5		Organisms mostly disintegrated; an occasional Gram-negative organism seen.
9			5			5		Organisms have disintegrated and disappeared.

ard solutions or bouillon without cellular material. An enzyme derived from the cellular material may also, however, be a factor in the dissolution.

Dissolution Experiment with Other Organisms.—To a cellular suspension from a Type I pneumonic lung with a pH of 5.5, suspensions

of living and washed *Streptococcus hæmolyticus* and of *Streptococcus viridans* were added. To another portion of the same cellular suspension, Type I pneumococcus was also added as a control. In setting up the experiment 5 drops of the cellular suspension were added to 5 drops of a suspension of the different organisms. Microscopic examination of the mixtures after incubation over night showed that the pneumococci had disappeared with the exception of an occasional Gram-positive organism. There were many fragments of Gram-positive organisms and many staining points. *Streptococcus hæmolyticus* and *Streptococcus viridans* were not dissolved and remained Gram-positive after exposure in the incubator to the cellular suspension.

CONCLUSIONS.

Pneumococci of Types I, II, and III undergo dissolution when mixed with cellular material from the pneumonic lung at a pH of 6.95 and 5.5, but not at a pH of 4.5. An enzyme derived from the bacteria themselves or from the cellular material may be the cause of the dissolution. *Streptococcus hæmolyticus* and *Streptococcus viridans* do not undergo dissolution under similar experimental conditions.

STUDIES ON THE PNEUMOCOCCUS.

IV. EFFECT OF BILE AT VARYING HYDROGEN ION CONCENTRATIONS ON DISSOLUTION OF PNEUMOCOCCI.

BY FREDERICK T. LORD, M.D., AND ROBERT N. NYE, M.D.

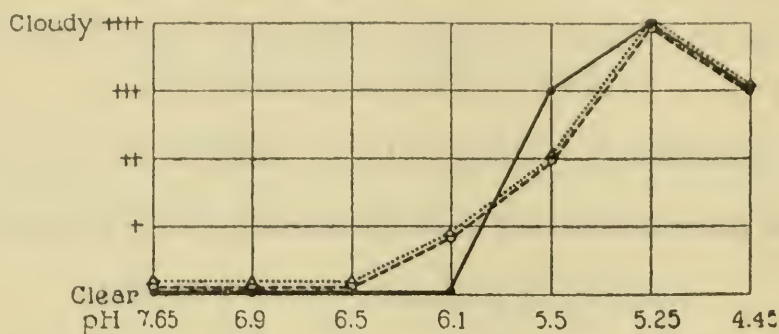
(From the Research Laboratory of the Massachusetts General Hospital, Boston.)

(Received for publication, December 16, 1921.)

Fresh beef bile, obtained from the slaughter house, after sterilization for 15 minutes in the autoclave, was found to have a pH of 7.8. By the addition of increasing amounts of HCl to different lots of this bile a series was made with a pH range of 7.65 to 4.45 as determined by the dialysis method (Table I). 4 drops from each of the series of tubes containing bile at varying pH were removed to four sets of seven test-tubes. To one set were added 4 drops of a suspension of *Pneumococcus* Type I, to a second an equal amount of a suspension of Type II, and to a third a suspension of Type III. The fourth set was reserved as a control, 4 drops of distilled water being added to each tube in place of the suspension of pneumococci. After incubation, inspection of the four different series showed the same result in all. As indicated in Text-fig. 1, the tubes containing the three fixed types of pneumococci showed clearing at pH 6.1 and the more alkaline end of the scale, and cloudiness in the more acid end of the scale. The control series without pneumococci showed cloudiness in the acid end of the scale due to precipitation of the bile solution at acidities greater than pH 6.1.

Microscopic examination of smears made from the cloudy tubes showed many masses of amorphous material and many pneumococci, for the most part Gram-negative, a considerable number of Gram-positive organisms, and the shadowy remains of others, while in the clear tubes only a few Gram-positive organisms were found with the shadowy remains of others.

Comparative tests with bile and standard pH solutions show that pneumococci undergo more rapid dissolution in bile. The dissolution in bile may be complete within an hour and only partial in standard solutions within this interval. Dissolution of pneumococci occurs



TEXT-FIG. 1. Dissolution of pneumococci in bile at varying hydrogen ion concentrations. Test-tubes containing 4 drops of bile at varying hydrogen ion concentrations plus 4 drops of suspension of living pneumococcus. Type I, solid line; Type II, dotted line; Type III, broken line. The observations were made after 24 hours in the incubator.

TABLE I.

Method of Preparing Bile at Varying Hydrogen Ion Concentrations.

Tube No.	Bile.	Amount of HCl (half concentrated).	pH	pH after sterilization, filtration, and resterilization.
		gtt.		
1	Sterile bile.	0	7.8	7.65
2	25 cc. of A.*	3		6.9
3	25 " " "	6	7.3	6.5
4	25 " " "	7		6.1
5	25 " " B.	6	5.85	5.5
6	25 " " "	10	5.35	5.25
7	C	0	4.9	4.45

* A, fresh bile at pH 7.8 after sterilization; B, bile at pH 6.85, obtained by adding 20 drops of concentrated HCl to 100 cc. of A; C, bile at pH 4.9, obtained by adding 80 drops of concentrated HCl to 100 cc. of A.

most rapidly in the more alkaline end of the bile scale and with the lapse of time is progressive toward the more acid end, becoming complete within 24 hours at a pH of about 6.0 to 7.8 inclusive. Dissolution in standard solutions, however, takes place most quickly at a pH

of about 5.0 to 7.0 and then progresses toward the more alkaline end of the scale. As in standard solutions, dissolution in bile does not take place at the most acid end of the scale.

Dissolution of pneumococci takes place in human as well as beef bile and at the same range of pH. Pneumococci allowed to grow and die out in glucose bouillon, washed, and suspended in bile at varying pH show some but less dissolution than when living organisms are used in the experiment. Bile solubility of dead organisms takes place within the usual range of pH but more slowly than when living organisms are used.

DISCUSSION.

The solubility of the pneumococcus in bile and in standard solutions may be ascribed to the liberation of an enzyme from the bacterial cell. The more rapid solubility of pneumococci in bile at a slightly alkaline reaction and in standard solution at a slightly acid reaction probably depends on a difference in the physical state of the bacterial cell under the influence of the two media. Bile itself probably has the property of killing the pneumococcus with a minimum of injury to the bacterial cell membrane and thus enables the endoenzymes to operate at their optimum reaction which Avery and Cullen¹ have shown to be between pH 7.0 and 7.8. Dissolution in standard solutions on the other hand, proceeds most rapidly at a slightly acid reaction because in this medium death of the organisms, which is dependent on the concentration of hydrogen ions, takes place at this reaction with a minimum of injury to the bacterial cell membrane. The more rapid dissolution in bile than in standard solutions is probably due to the more rapid death of the organisms in bile and hence the liberation of a larger amount of enzyme at the optimum pH in bile than in standard solution.

CONCLUSION.

Dissolution of pneumococci takes place most rapidly in bile at a slightly alkaline reaction. This is probably due to death of the organisms and activation of the endocellular enzyme at its optimum hydrogen ion concentration.

¹ Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 547, 571, 583.

SURFACE TENSION OF SERUM.

II. ACTION OF TIME ON THE SURFACE TENSION OF SERUM SOLUTIONS.

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PLATES 58 TO 61.

(Received for publication, January 11, 1922.)

I.

INTRODUCTION.

It has been shown in a previous paper¹ that the surface tension of serum decreased rapidly, as soon as the serum was exposed to the air. This phenomenon is due to the adsorption, in the surface layer, of substances which either travel slowly in the liquid, or are formed in the surface layer, or else to modifications in the arrangements of the group molecules in the surface layer, and possibly to a combination of these causes. In order to study these substances and to obtain an idea of the order of magnitude of their power of lowering the surface tension of water in function of time, it was necessary to measure the surface tension of the same samples of solutions of serum at different intervals, ranging from 2 minutes to 24 hours.

II.

EXPERIMENTAL.

1. *Decrease of Surface Tension of Serum Solutions in Function of Time.*—Solutions of fresh serum were made in saline solution (NaCl 0.9 per cent). The surface tension of the same layer of liquid was measured by means of du Noüy's tensiometer, according to the technique previously described.¹ In order to prevent any jarring of the samples studied, a turntable was made, supported on a ball thrust bearing, on which ten watch-glasses were placed in a circle. By

¹ du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.

turning the table carefully, the watch-glasses were brought successively under the ring, and then raised smoothly by means of the screw of the instrument until the contact was established. The results obtained are shown in Table I.

Higher dilutions were prepared and tested after 1, 2, 20, and 23 hours, with the results shown in Tables II and III.

This phenomenon is illustrated by Text-fig. 1. In the charts, the value of the surface tension is taken after 2 hours arbitrarily, but the maximum drop occurs in the first 30 minutes (Text-fig. 2). The lapse of 2 hours was chosen because after that period of time, the drop is usually very slow. Text-figs. 3 and 4 show that between 1 and 2 hours the drop is small.

The drop in surface tension may be expressed in two different ways according to whether one chooses to take into account either the initial value of the surface tension as measured after a few seconds, or else to assume that the drop is so rapid at the beginning that a difference of 1 second or less is sufficient to affect the initial reading. In the latter case, the initial value may be taken as equal to 76 dynes, roughly, and the differences expressed thus:

$$-d\gamma = 76.0 - \gamma$$

The initial values and the values after 2 hours are shown in Text-figs. 3 to 9. The drop, $-d\gamma = \gamma_0 - \gamma$, is expressed in Text-figs. 10 to 15, and the drop, $-d\gamma = 76.0 - \gamma$, in Text-fig. 16.

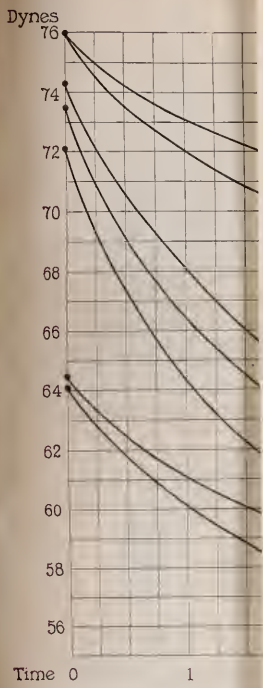
2. *Comparison with the Behavior of Some Organic Salts.*—It may be of interest to compare the action of serum with that of substances having a strong effect on the surface tension of water, such as sodium oleate, sodium glycocholate, and saponin, at similar dilutions in the same saline solution (Table IV). The phenomenon is illustrated in Text-figs. 17 to 19. Comparison with Text-figs. 3 to 9 shows the likeness to the behavior of serum, although the initial value is much higher in the latter case. This fact is probably due to substances which have some kind of buffing action, and oppose the lowering of surface tension of serum. The substances are particularly active when a foreign body, such as oleate or glycocholate, is added to the serum. This phenomenon will be studied in another paper.²

² du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv (in press).

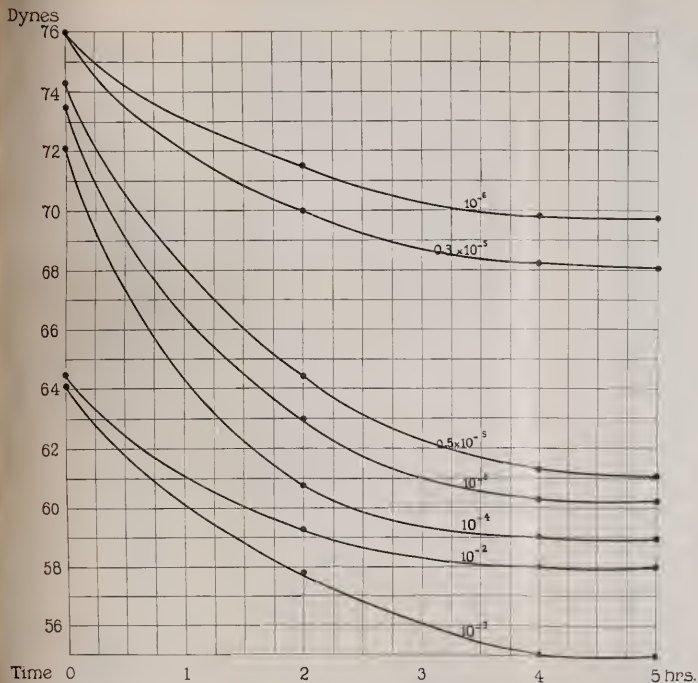
Faldout

70

708^g



TEXT-FIG. 1. Values of the surface



TEXT-FIG. 1. Values of the surface tension of serum solutions in function of the time. Serum 118 (rabbit).

TABLE I.

Decrease of Surface Tension of Serum Solutions at Different Concentrations in Function of Time.

*Fresh Rabbit Serum, No. 114 (Text-Fig. 2).
Temperature 22° C.*

Time.....	0	2 min.	6 min.	10 min.	30 min.	2 hrs.	Drop in 2 hrs.
Pure.	65.5*	64.5	64.0	64.0		63.0	2.5
Dilution.							
1:100	69.0	67.0			64.0	59.3	9.7
1:1,000	67.0	65.0			62.5	53.0	14.0
1:5,000	71.6	69.5			58.0	52.6	19.0
1:10,000	72.0	68.1	64.6	63.0	60.0	56.0	16.0
1:20,000	75.5	73.0			67.5	61.0	14.5
1:30,000	75.0	74.0			67.0	63.0	12.0
1:40,000	72.2	70.0			67.2	62.0	10.0
1:50,000	75.8	75.8			70.0	65.6	10.2
1:100,000	68.0	68.0			62.0	56.9	11.1
1:200,000	73.5	73.0			69.0	62.0	10.5

* In all the tables the determinations are given in dynes.

TABLE II.

Decrease of Surface Tension of Serum Solutions at Different Concentrations in Function of Time.

*Fresh Rabbit Serum, No. 114.
Temperature 22° C.*

Time.....	0	1 hr.	2 hrs.	20 hrs.	23 hrs.	Drop in 23 hrs.
Dilution.						
1:400,000	67.0	76.2	67.0		64.0	12.0
1:800,000	76.2	73.2	70.7		67.0	9.2
1:1,000,000	76.0		69.5	70.1		6.0
1:3,000,000	73.5	71.5		69.0		4.5
1:5,000,000	72.1			70.1		2.1
Control.						
Pure saline solution, 0.9 per cent.....	75.3				73.5	1.8
2nd sample.	76.5				75.6	1.5
3rd " covered...	76.2				76.2	0

TABLE III.

Decrease of Surface Tension of Serum Solutions at Different Concentrations in Function of Time.

Fresh Rabbit Serum, No. 116 (Text-Fig. 3).

Temperature 20° C.

Dilution.....	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	68.3	71.0	73.5	76.0	76.5
After 2 hrs.....	62.0	61.8	62.0	65.9	72.0
“ 5 “	58.9	57.0	57.5	64.0	71.5

Fresh Rabbit Serum, No. 117 (Text-Fig. 5).

Temperature 20° C.

Dilution.....	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	66.1	66.5	72.9	76.0	76.3
After 2 hrs.....	63.0	63.0	64.5	68.2	73.2
“ 5 “	60.0	59.2	59.0	63.0	71.4

Fresh Dog Serum, No. 1 (Text-Fig. 6).

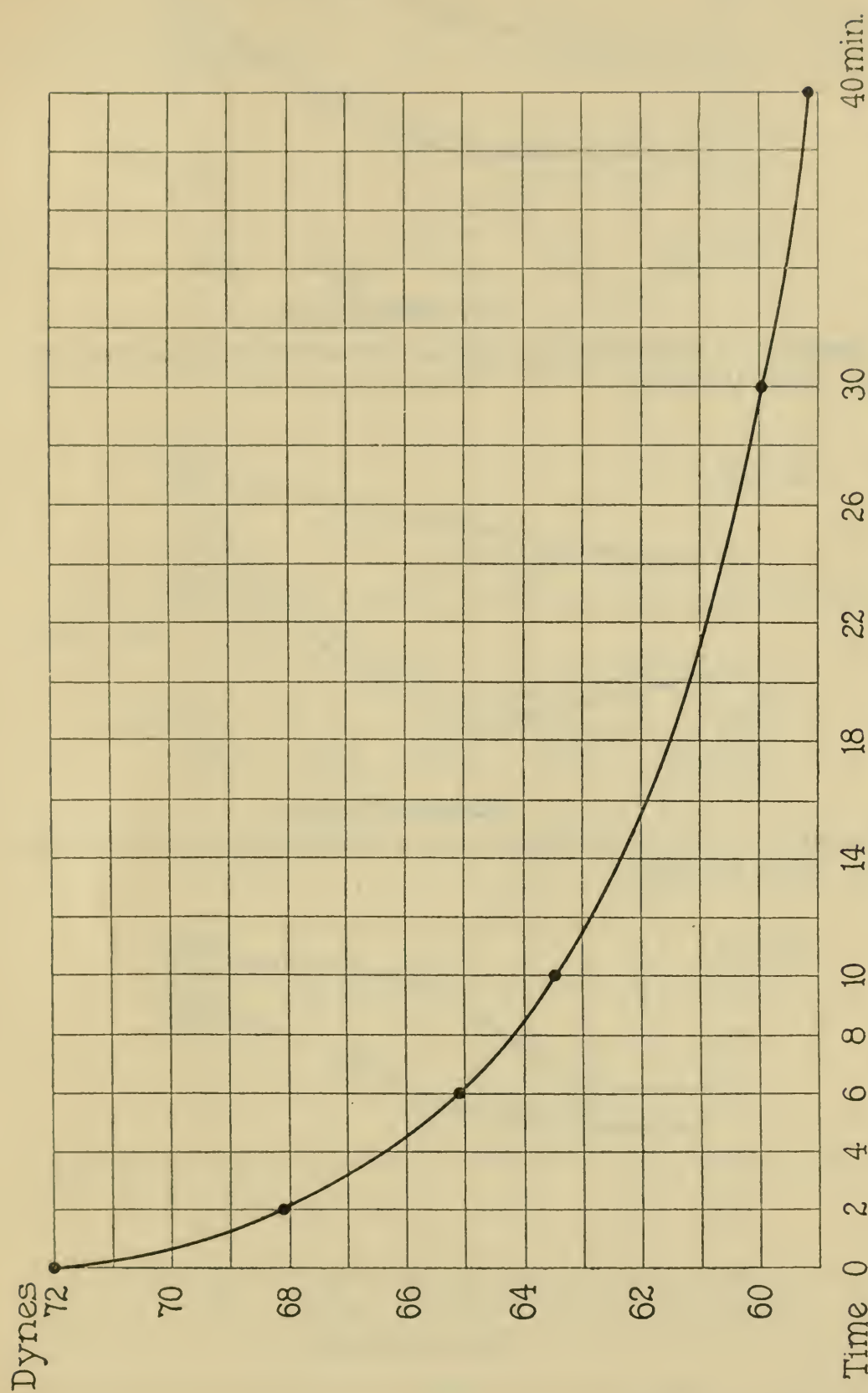
Temperature 20° C.

Dilution.....	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	63.5	66.0	73.0	75.0	76.0
After 3 hrs.....	58.0	57.3	59.0	62.5	70.0

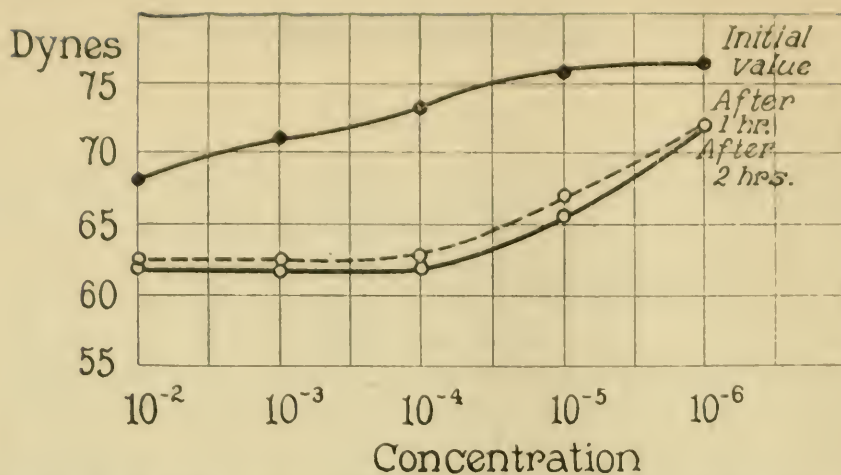
Fresh Human Serum (Text-Fig. 8).

Temperature 22° C.

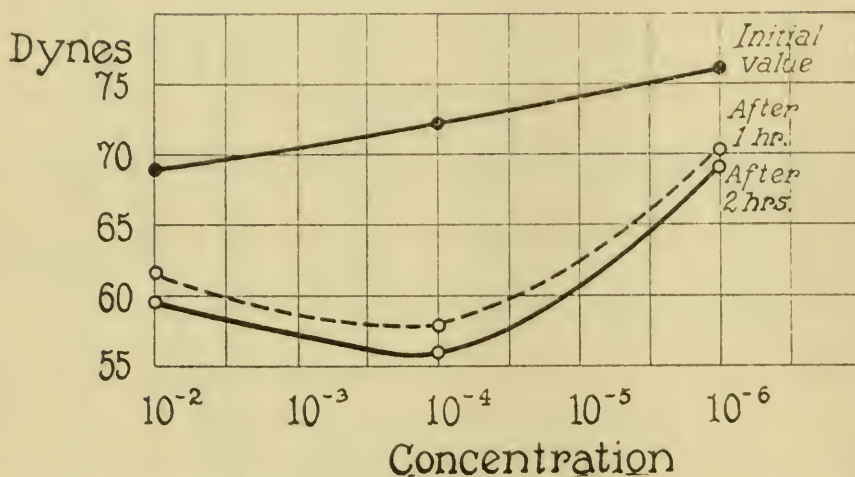
Dilution.....	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	61.1	65.0	72.0	76.0	76.0
After 2 hrs.....	55.5	57.0	58.0	61.9	73.0



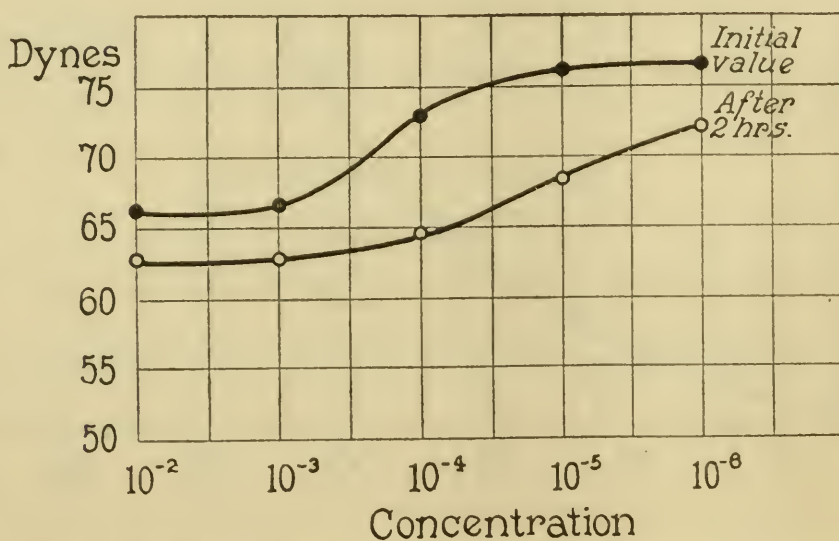
TEXT-FIG. 2. Values of the surface tension of a serum solution in the first 40 minutes. Dilution 1:10,000. Serum 114 (rabbit).



TEXT-FIG. 3. Values of the surface tension of serum solutions in function of the time. Serum 116 (rabbit).

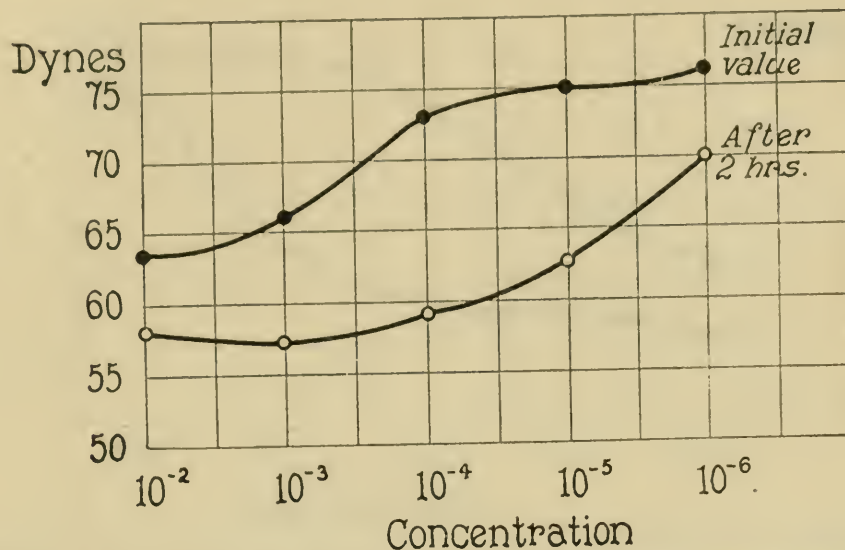


TEXT-FIG. 4. Values of the surface tension of serum solutions in function of the time. Serum 114 (rabbit).

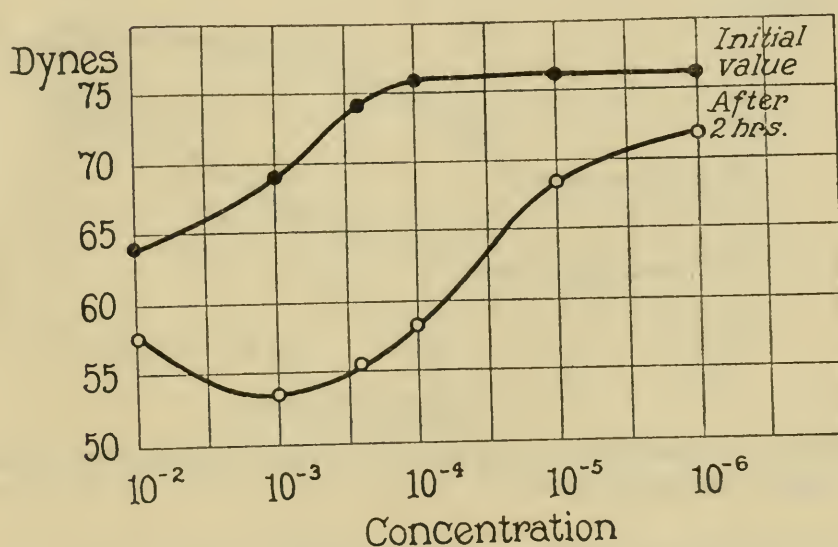


TEXT-FIG. 5. Values of the surface tension of serum solutions in function of the time. Serum 117 (rabbit).

The decrease of surface tension of solutions of organic compounds, such as glycocholate for example, follows the same law as that of serum



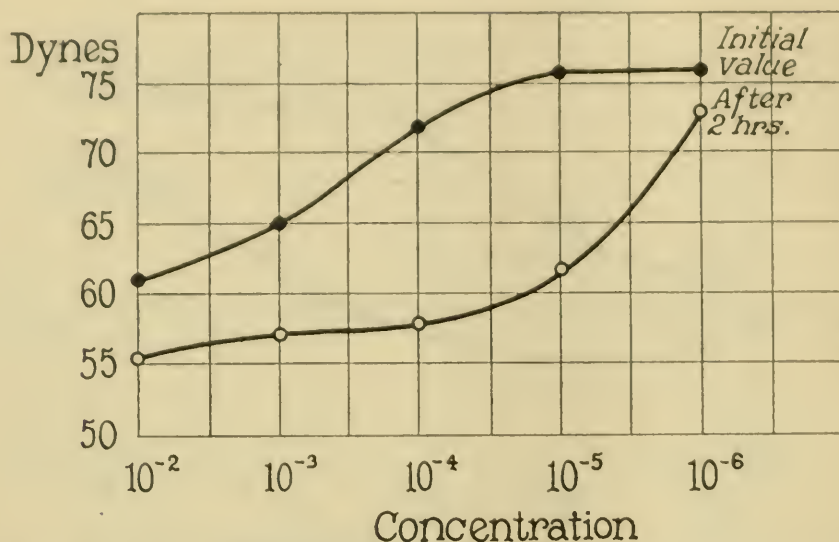
TEXT-FIG. 6. Values of the surface tension of serum solutions in function of the time. Serum 1 (dog).



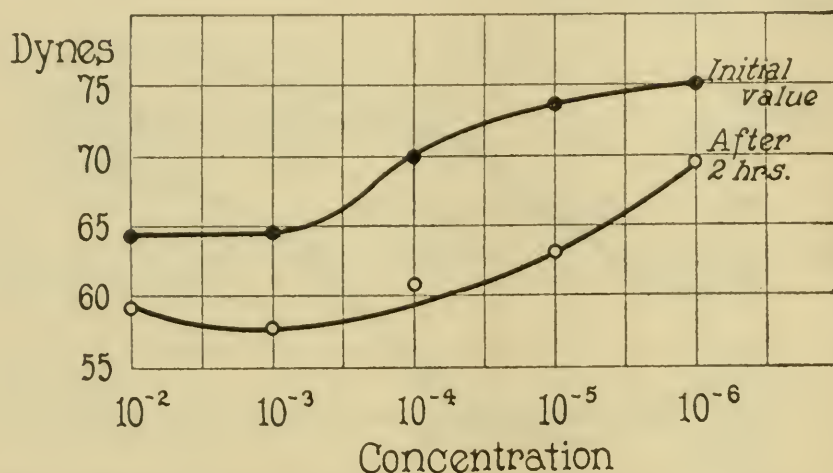
TEXT-FIG. 7. Values of the surface tension of serum solutions in function of the time. Young dog serum.

and is also very rapid at the beginning (Text-fig. 20). In this case the glycocholate was dissolved in distilled water (concentration 10^{-4}). When serum is diluted in distilled water, the same phenomenon occurs

(Text-figs. 21 and 22). The maxima and the comparison with serum are shown in Text-figs. 23 and 24.



TEXT-FIG. 8. Values of the surface tension of serum solutions in function of the time. Human serum.

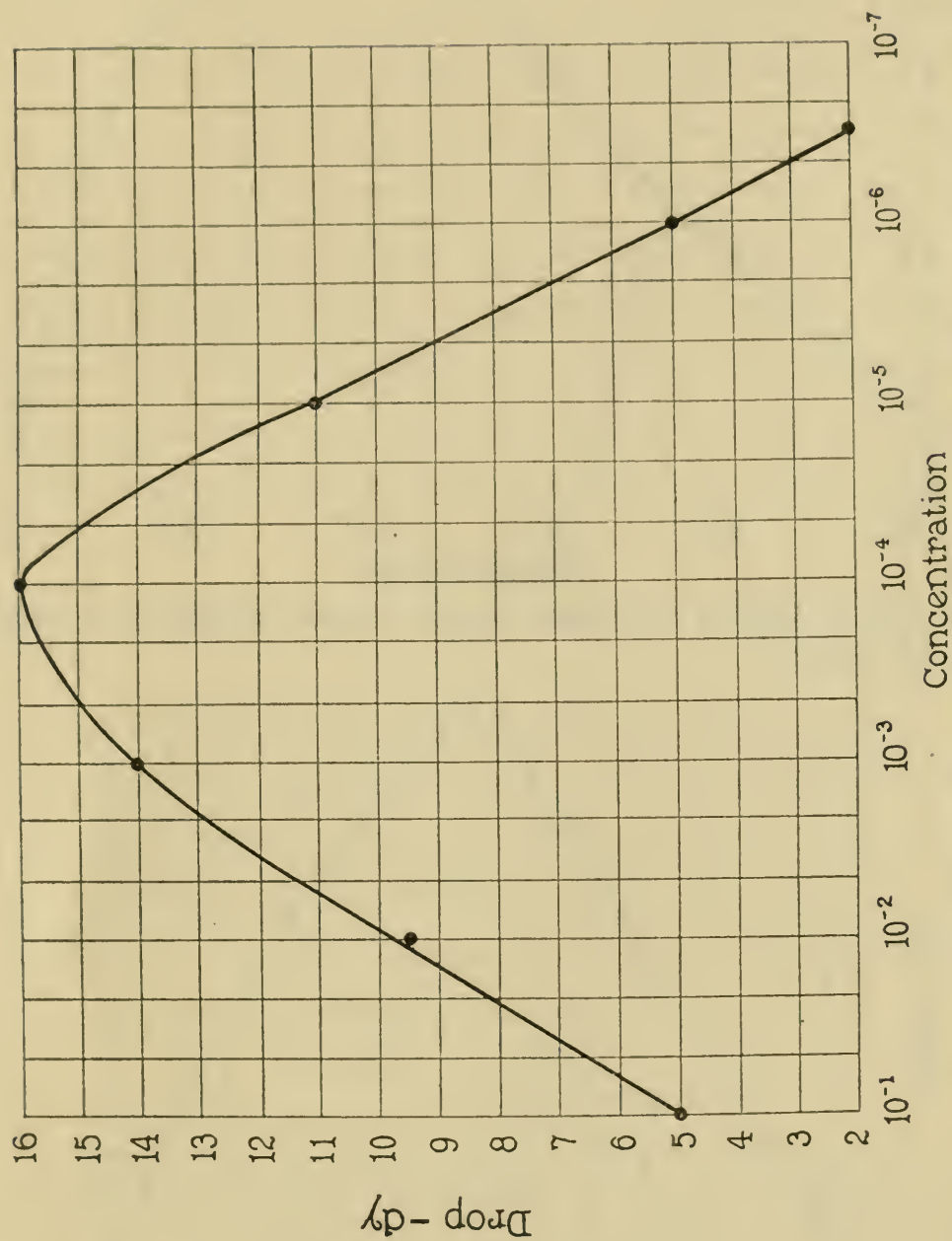


TEXT-FIG. 9. Values of the surface tension of serum solutions in function of the time. Serum 118 (rabbit).

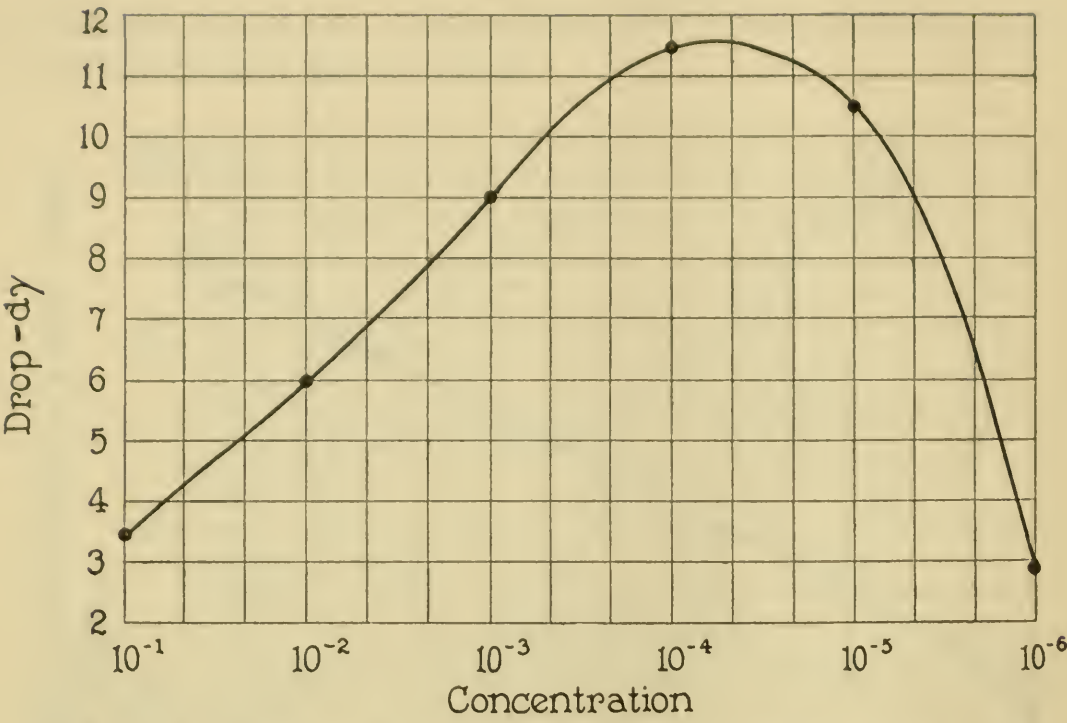
It may be of interest to refer the reader to the work of Berczeller³ on the surface tension of colloidal solutions and, in general, to the numerous papers published by Traube on the same subject. The book by Freundlich,⁴ a great number of papers

³ Berczeller, L., *Biochem. Z.*, 1913, liii, 215; 1914, lxvi, 173; *Internat. Z. physik.-chem. Biol.*, 1914, i, 124.

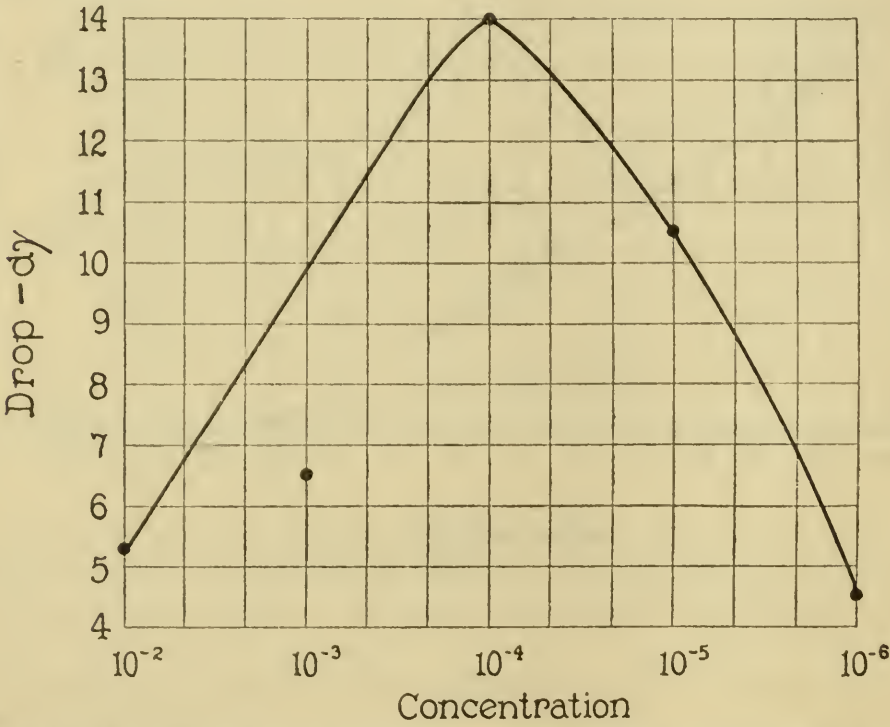
⁴ Freundlich, H., *Kapillarchemie: Eine Darstellung der Chemie der Kolloide und verwandter Gebiete*, Leipsic, 1909.



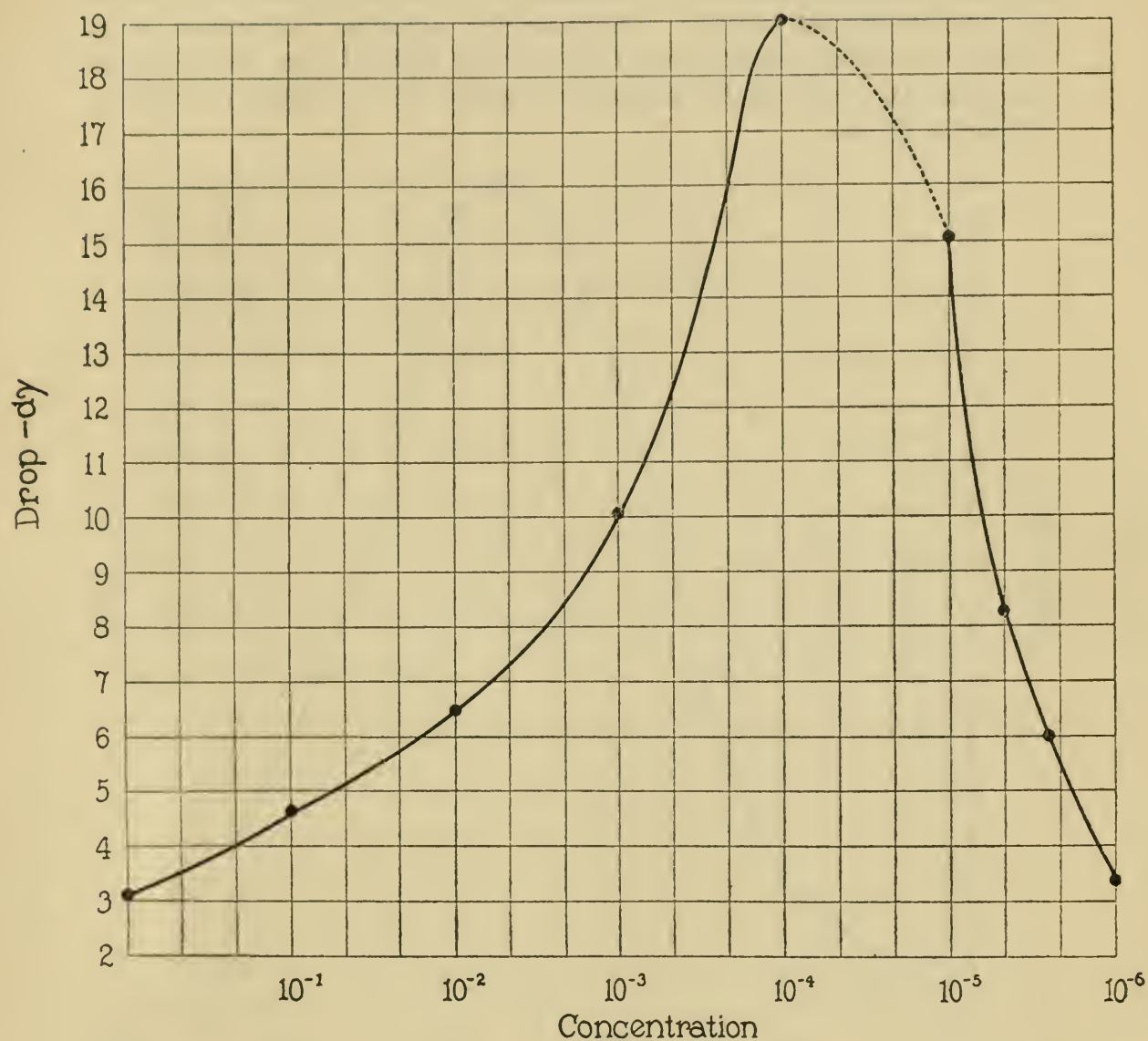
TEXT-FIG. 10. Drop in the surface tension of serum solutions in 2 hours. Serum 114 (rabbit).



TEXT-FIG. 11. Drop in the surface tension of serum solutions in 2 hours. Serum 116 (rabbit).

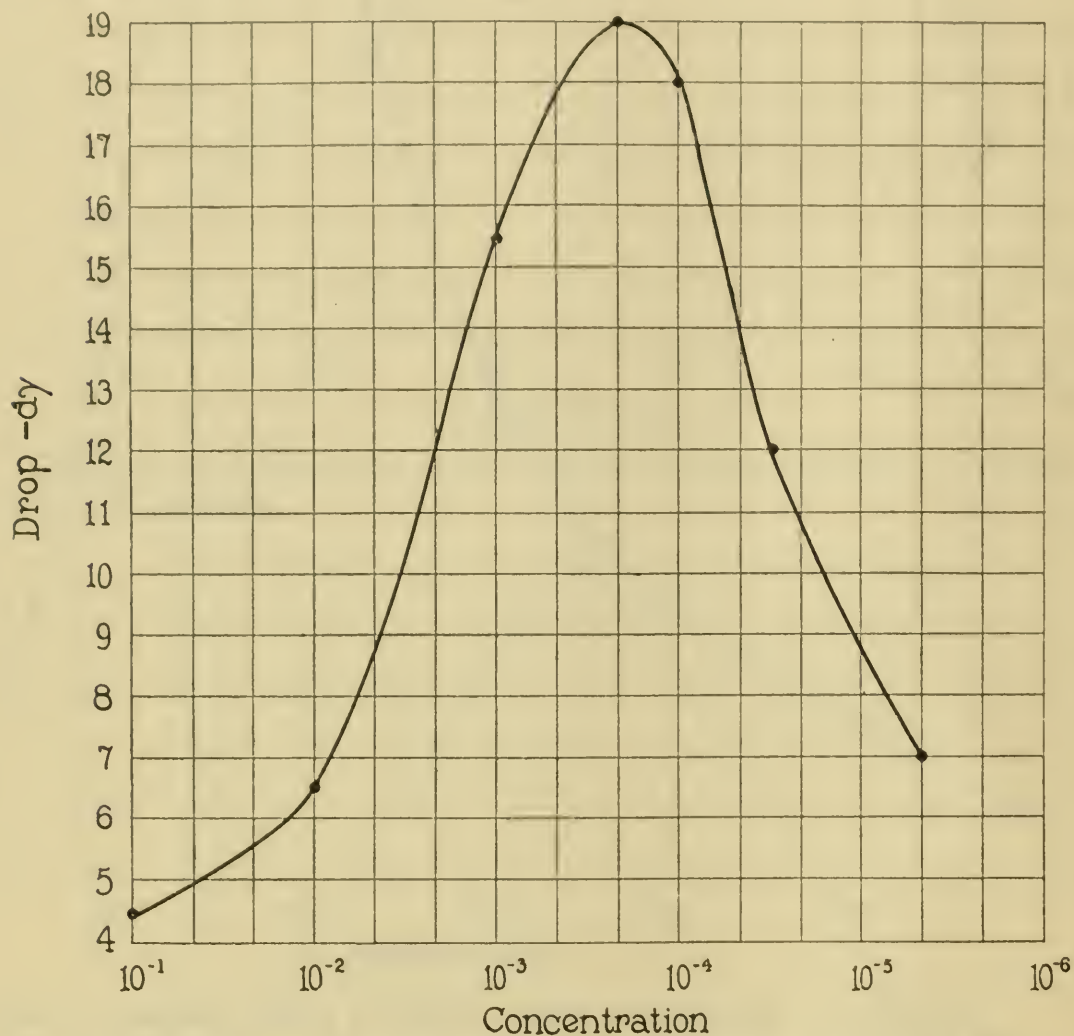


TEXT-FIG. 12. Drop in the surface tension of serum solutions in 2 hours. Serum 118 (rabbit).



TEXT-FIG. 13. Drop in the surface tension of serum solutions in 2 hours. Old dog serum. As no measurements were made at concentrations between 10^{-4} and 10^{-5} a dotted line is used to connect these two points, since it is probable that the maximum would have been somewhere between these two concentrations. Therefore it is likely that the dotted line does not represent the exact curve.

in the *Internationale Zeitschrift für physikalisch-chemische Biologie*, and the papers by Posternak⁵ are also worth perusal. It seems that the solution of the problem has been postponed mainly by the confusion of the action of time with that of heat and of chemical reactions. Therefore, it is important to determine, in all studies on colloidal solutions, the part due to their colloidal state and that which may be due to really specific actions (for instance the inactivation of serum by heating at 56° C.).

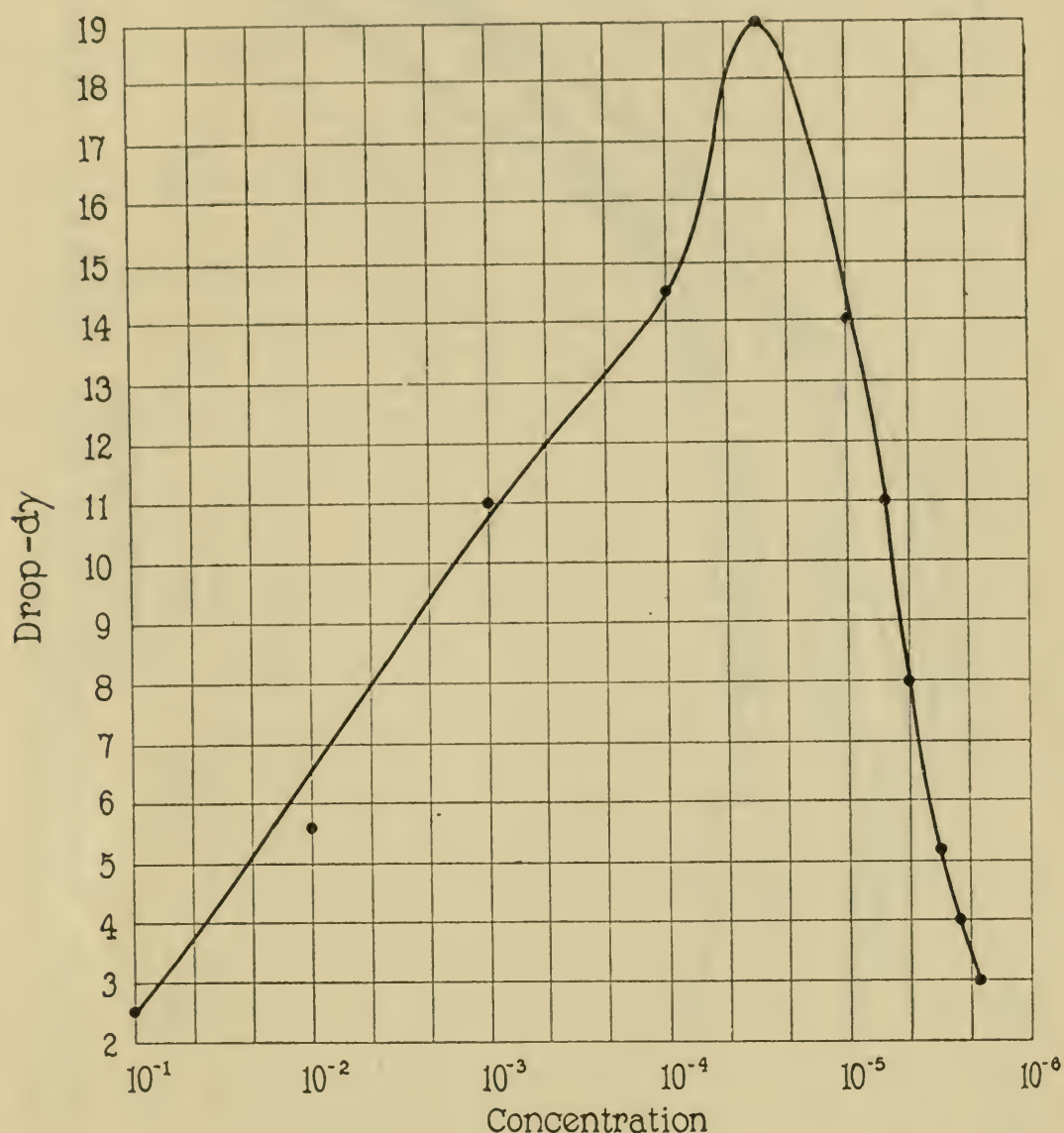


TEXT-FIG. 14. Drop in the surface tension of serum solutions in 2 hours. Young dog serum.

Thus, by merely introducing the factor time, are explained some of the discrepancies hitherto unaccounted for between the values of surface tension of solutions of organic compounds, as given by static

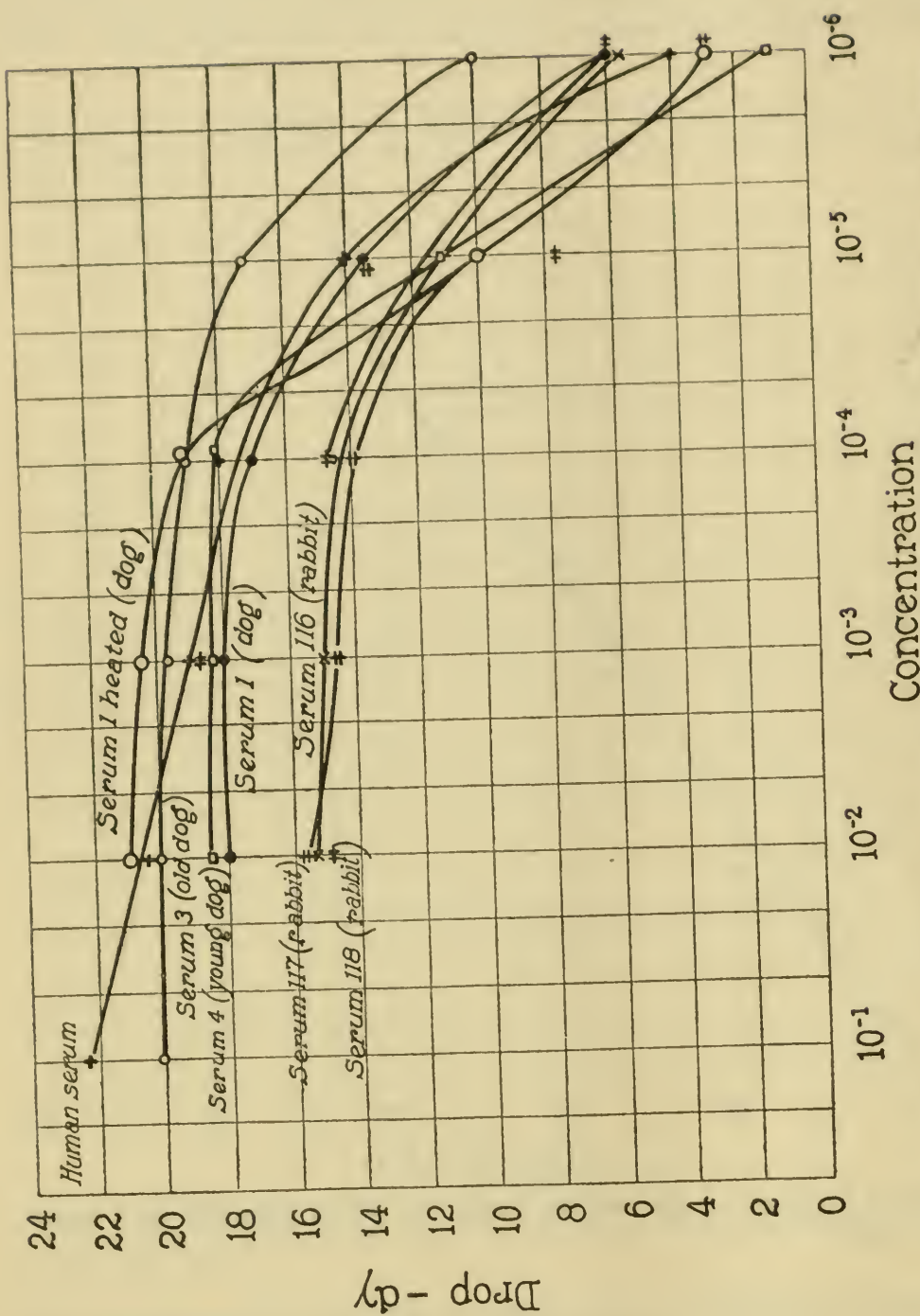
⁵ Posternak, S., *Ann. Inst. Pasteur*, 1901, xv, 85, 169, 451, 570.

and dynamic methods. Indeed, certain methods, such as the capillary ascension for instance, measure the surface tension of the same layer of liquid in which adsorption in function of the time takes place, while



TEXT-FIG. 15. Drop in the surface tension of serum solutions in 2 hours. Human serum.

most dynamic methods, such as the undulatory jet methods, deal with a continuously renewed surface. The drop weight method is intermediate, the drops requiring a longer time to fall. It is quite obvious that none of these last methods could show the drop in



TEXT-FIG. 16. Drop in the surface tension of solutions of different sera in 2 hours.
Value after 2 hours subtracted from 76.0 dynes.

TABLE IV.

Decrease of Surface Tension of Solutions of Sodium Oleate, Sodium Glycocholate, and Saponin in a 0.9 Per Cent Saline Solution in Function of Time.

Sodium Oleate (Text-Fig. 17).

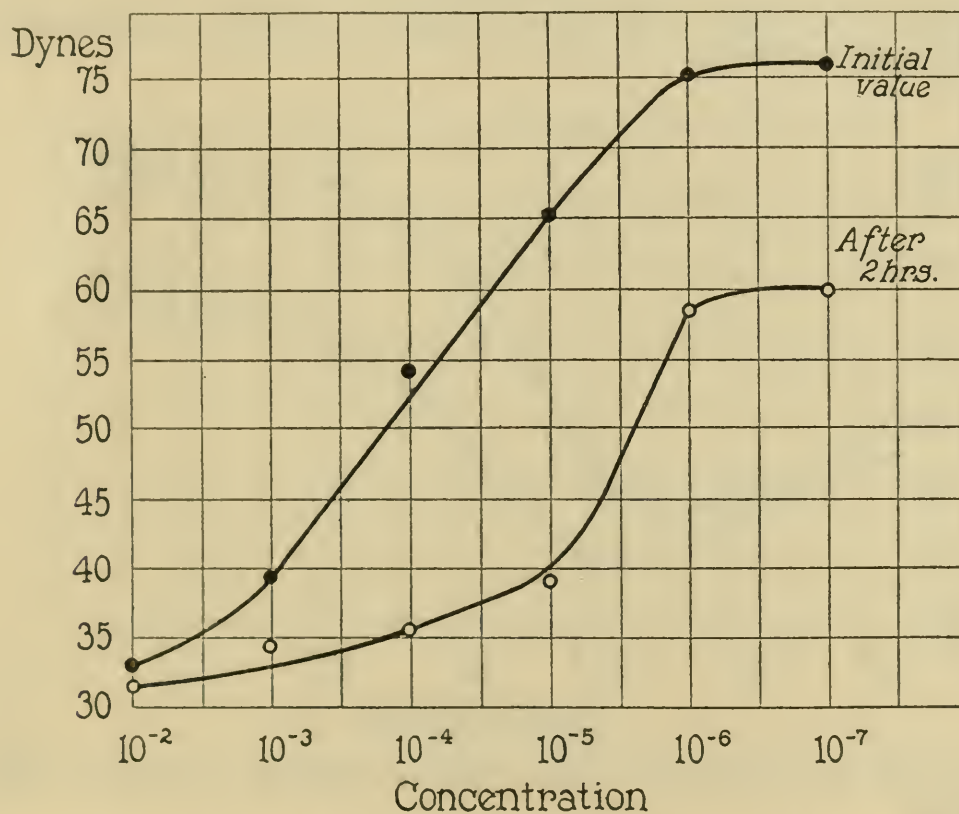
Dilution.....	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	33.2	39.5	54.2	59.1	75.1
After 2 hrs.....	31.5	34.5	35.5	39.0	58.6

Sodium Glycocholate (Text-Fig. 18).

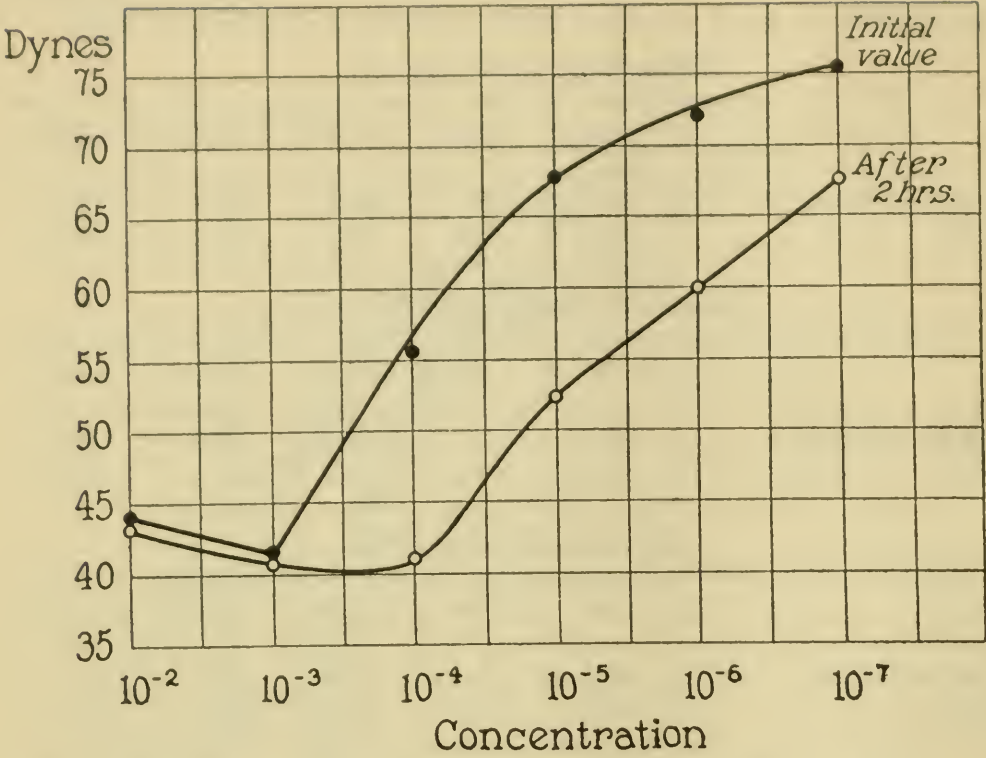
Dilution.....	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	44.0	41.5	55.6	68.0	72.0
After 2 hrs.....	43.4	41.0	41.5	60.6	60.0

Saponin (Text-Fig. 19).

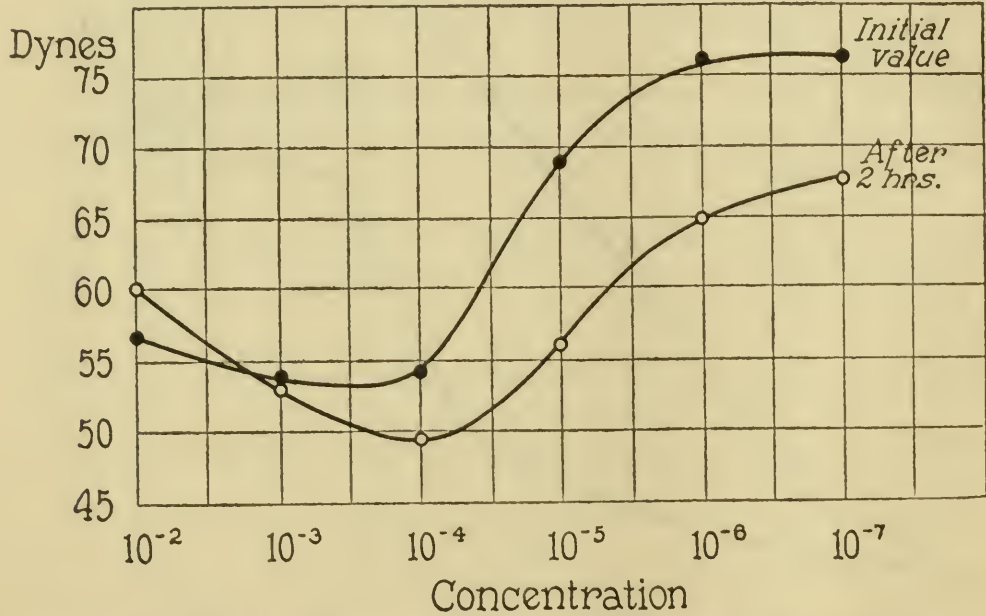
Dilution.....	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Initial surface tension	56.8	54.0	54.2	69.0	76.0
After 2 hrs.....	60.5	53.5	49.5	56.0	65.0



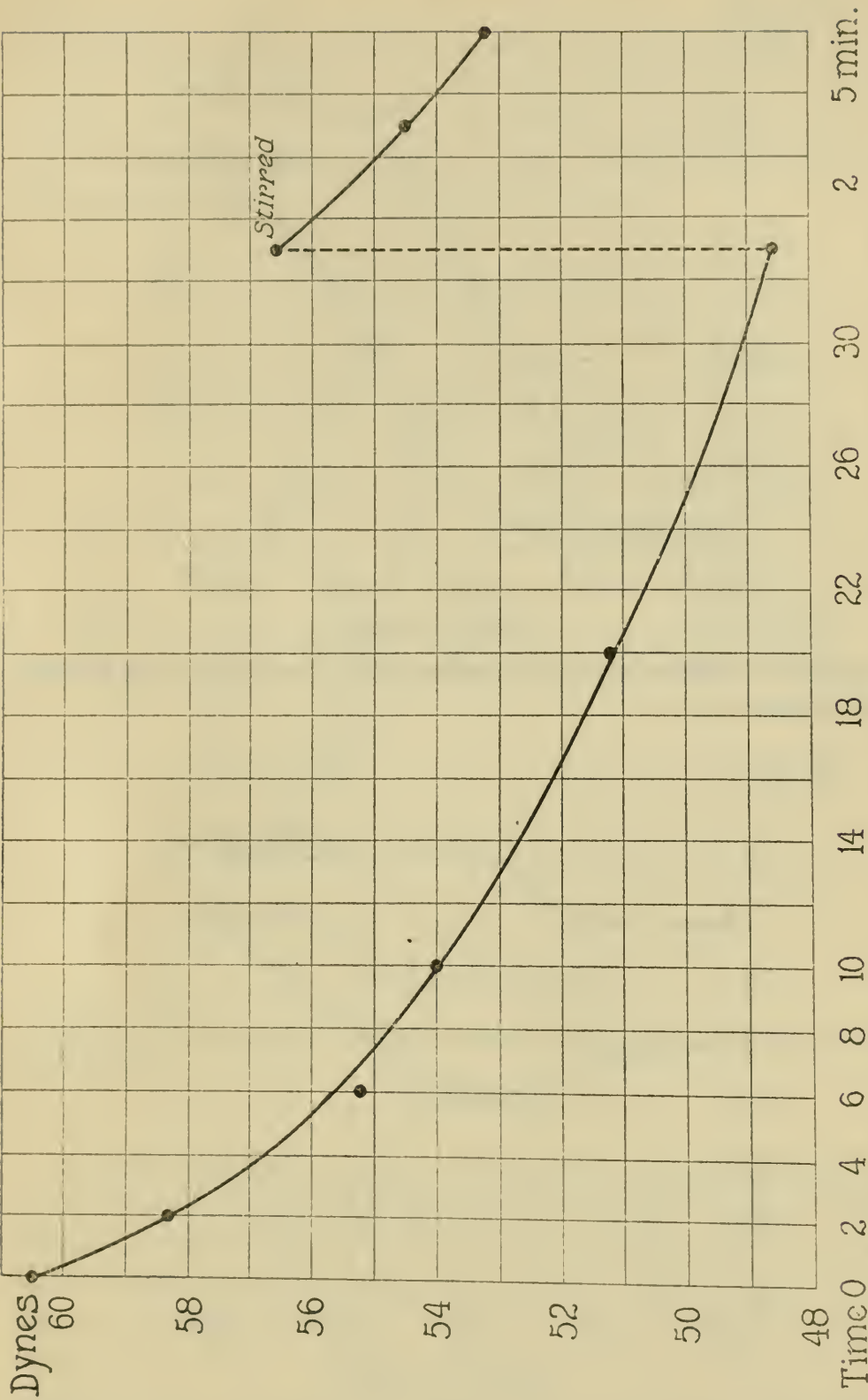
TEXT-FIG. 17. Values of the surface tension of solutions of sodium oleate in saline solution.



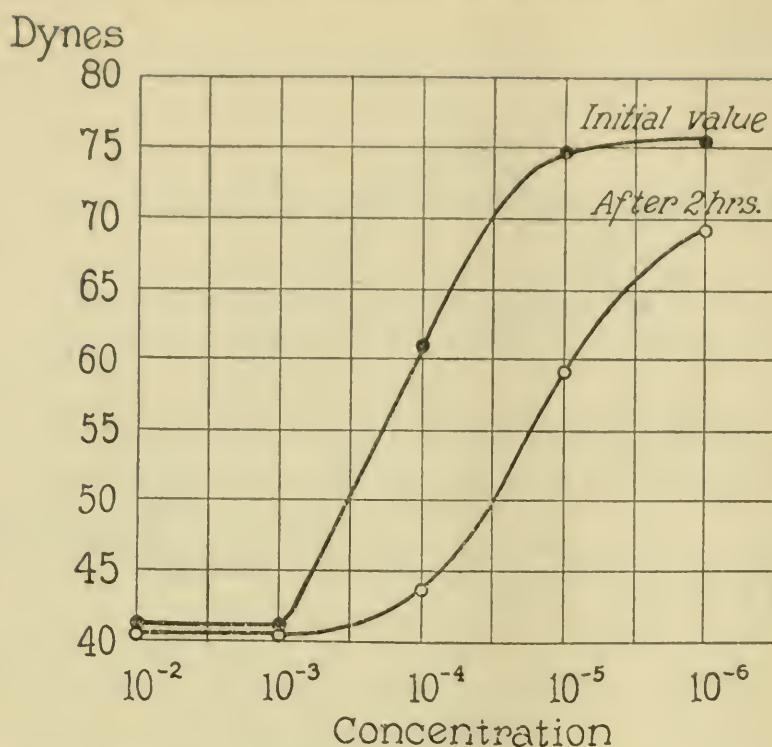
TEXT-FIG. 18. Values of the surface tension of solutions of sodium glycolate in saline solution.



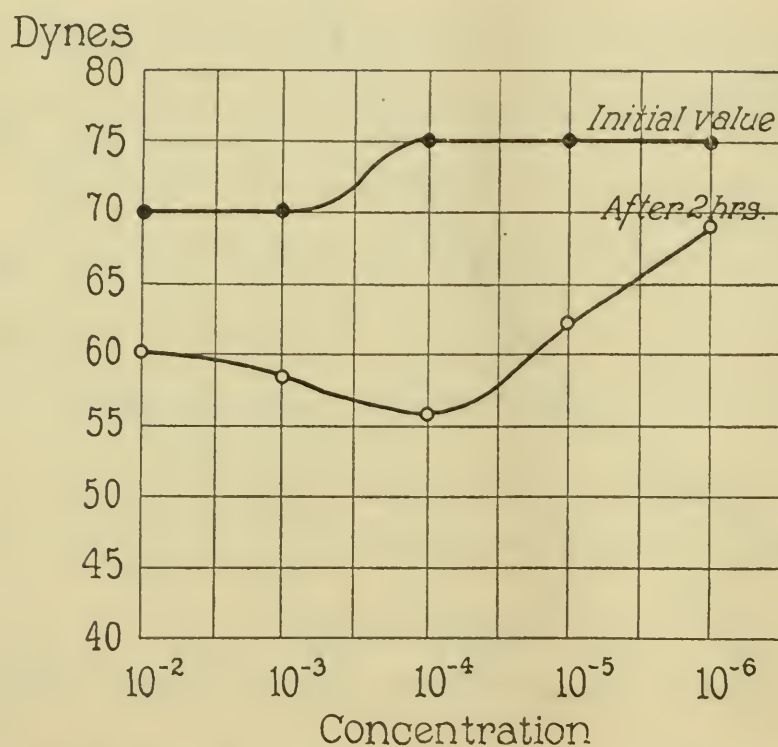
TEXT-FIG. 19. Values of the surface tension of solutions of saponin in saline solution.



TEXT-FIG. 20. Values of the surface tension of solutions of sodium glycocholate in distilled water. Drop in the first 30 minutes; effect of stirring. Concentration 1:10,000.

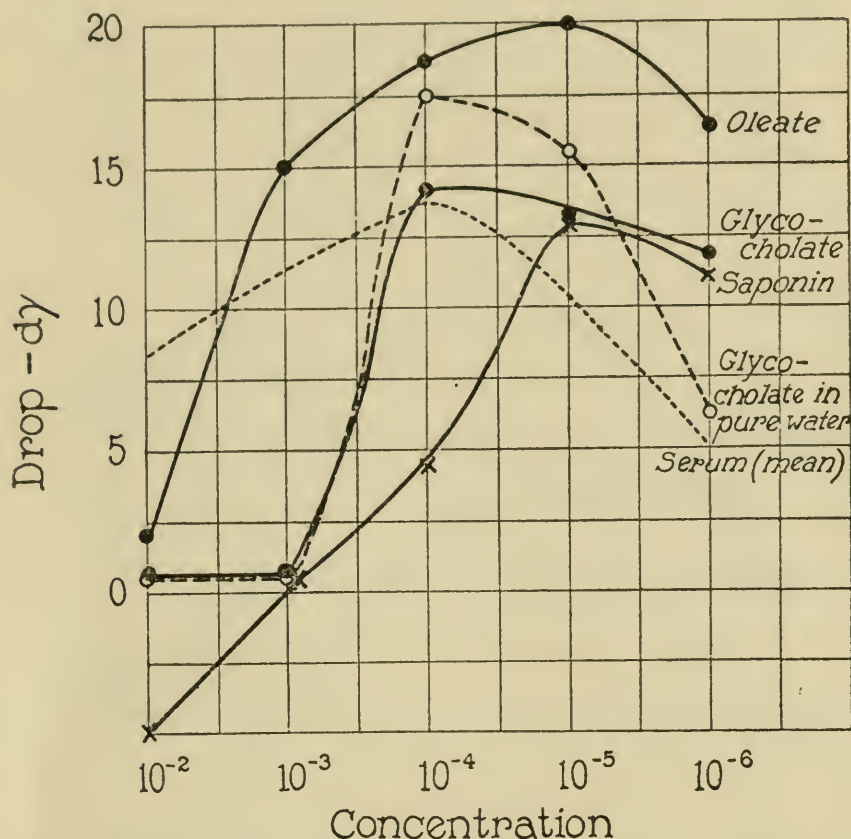


TEXT-FIG. 21. Values of the surface tension of solutions of sodium glycocholate in distilled water.



TEXT-FIG. 22. Values of the surface tension of solutions of serum in distilled water. Serum 118 (rabbit).

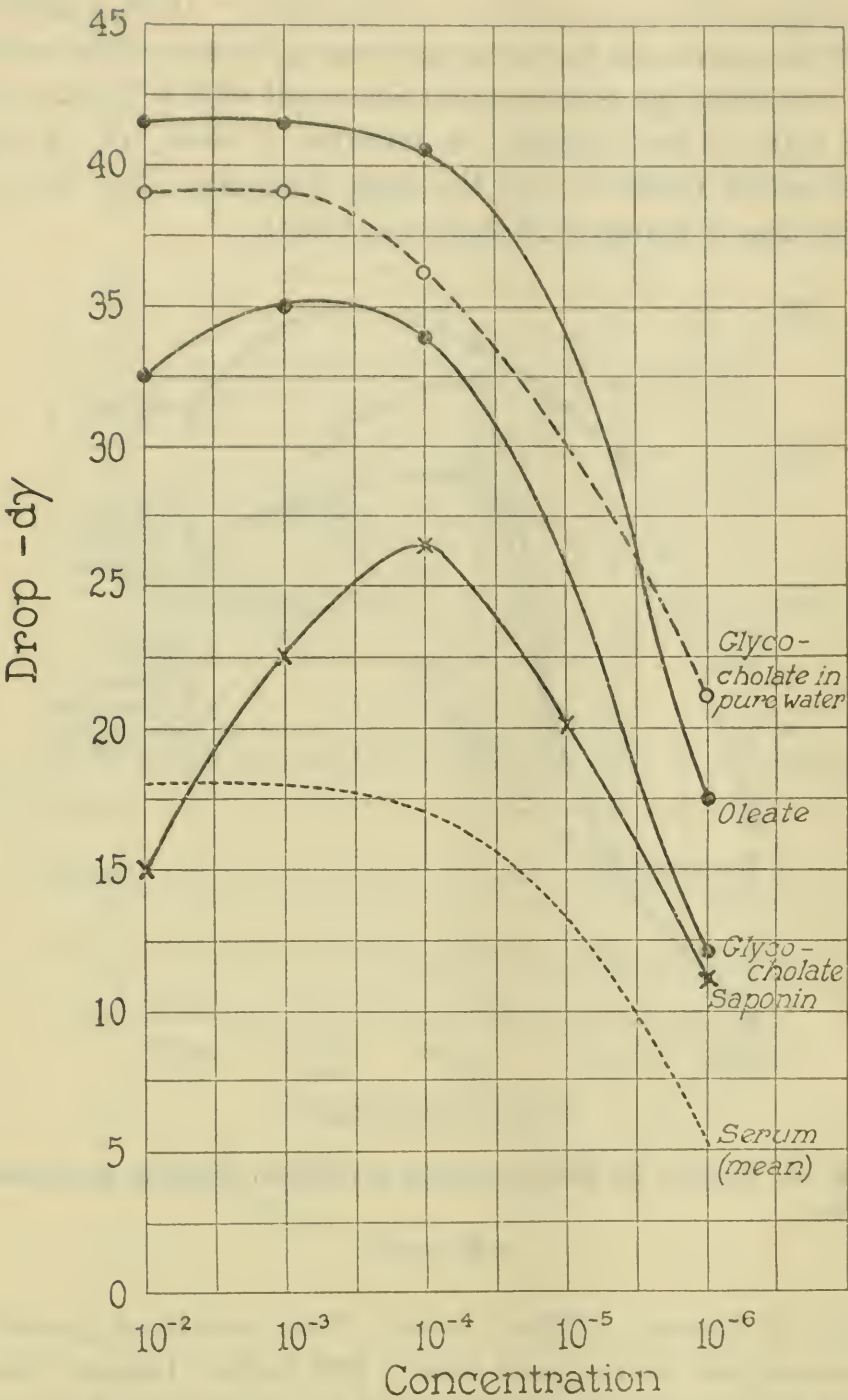
surface tension which occurs after a few minutes. With a solution of 1:100,000 the number of drops was the same as for pure saline solution: 17 drops whenever the measurement was made with a Traube stalagmometer (after a few seconds, 10 minutes, 2 hours, or 24 hours). The tensiometer measured for the same intervals: 75.5, 66.0, 61.0, 55.5 dynes; that is, a drop of 20 dynes in 24 hours.



TEXT-FIG. 23. Drop in the surface tension of different solutions as expressed by the formula

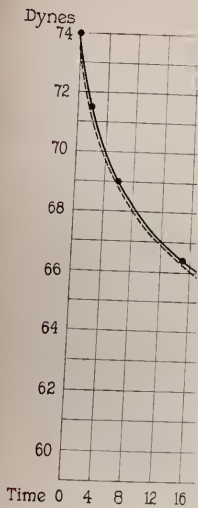
$$-d\gamma = \gamma_0 - \gamma$$

3. *Effect of Stirring.*—When stirred, these solutions present the same phenomenon as the pure serum; the surface tension rises and sometimes reaches the same value as at the beginning of the experiment (Table V and Text-figs. 25 and 26). When the solutions are kept in test-tubes, that is with a relatively small free surface, vigorous stirring after 24 hours brings the surface tension to the same value (Table VI). This seems to indicate that the damping effect,

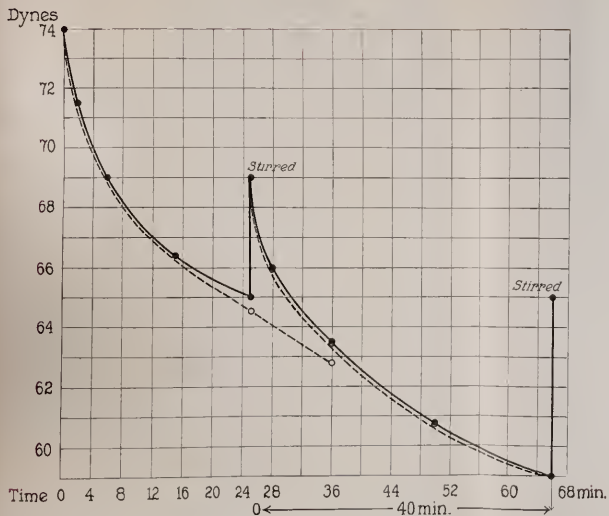


TEXT-FIG. 24. Drop in the surface tension of different solutions as expressed by the formula

$$-d\gamma = 76 - \gamma$$

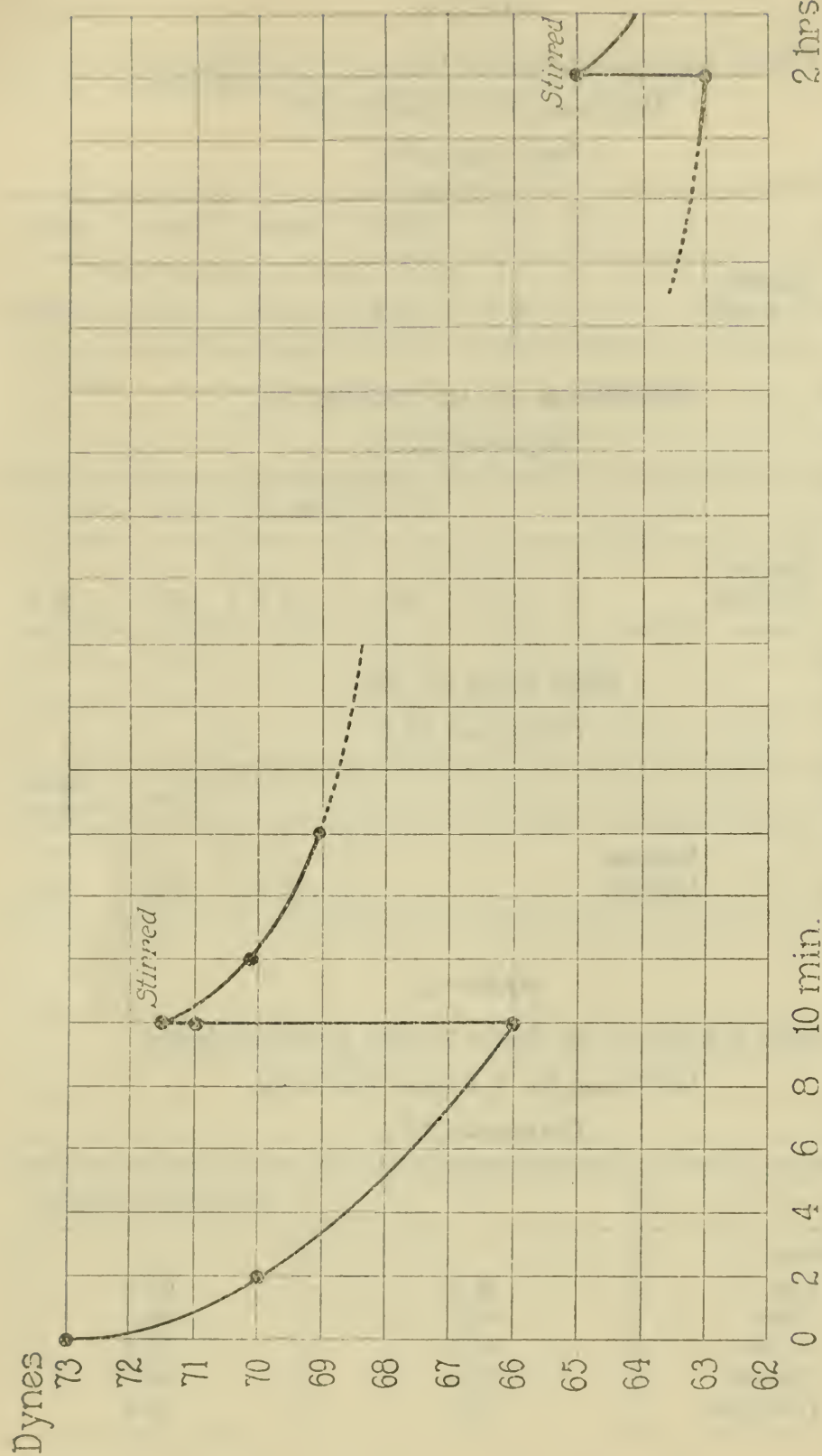


TEXT-FIG. 25. Effect o.
(dog). The dotted line is



TEXT-FIG. 25. Effect of stirring on solutions of serum. Dilution 1:10,000. Serum 2 (dog). The dotted line is calculated according to the formula

$$\gamma = \gamma_0 e^{-Kt}$$



Text-Fig. 26. Effect of stirring on solutions of serum. Dilution 1:10,000. Serum 112 (rabbit). The interruption of the curve after the 22nd minute was necessary to bring the end of the curve after 2 hours within the same chart, without making it too long.

TABLE V.

*Effect of Stirring on the Surface Tension of Serum Solutions.**Dog Serum, No. 2 (Text-Fig. 25).**Temperature 22° C.*

Time	0	25 min.	Stirred.	40 min.	Stirred.
Dilution. 1:10,000	74.0	64.9	69.0	59.0	65.0

*Rabbit Serum, No. 112 (Text-Fig. 26).**Temperature 22° C.*

Time	0	10 min.	Stirred.	2 hrs.	Stirred.
Dilution. 1:10,000	73.0	66.0	71.5	63.0	65.0

*Rabbit Serum, No. 118.**Temperature 23° C.*

Time	0	24 hrs.	Stirred vigorously.
Dilution. 1:10,000	76.0	68.2	75.1

TABLE VI.

*Effect of Stirring on the Surface Tension of Serum Solutions.**Dog Serum, No. 3, Kept in Test-Tubes.**Temperature 23° C.*

Time	0	24 hrs., stirred vigorously.
Dilution.		
1:10	61.9	62.0
1:100	62.5	60.0
1:1,000	66.2	66.0
1:10,000	76.0	76.0
1:100,000	74.0	74.0

described in our previous paper,⁶ is due to chemical changes undergone in the surface layer. When the surface layer is small with respect to the bulk, the changes are undetectable.

III.

DISCUSSION.

1. *Interpretation of the Maxima.*—From Table I, as well as from Text-figs. 10 to 15, it is clear that the drop in the value of the surface tension is variable and reaches a maximum at a dilution of about 1:10,000. In general, this occurs only when the measured initial value of the tension of the serum is taken into consideration. In other words, if we assume the initial value of the tension to be around 76 dynes, as in the higher dilutions, there would not always be a maximum. Therefore, we assume provisionally that the actually initial surface tension of such dilutions as 1:10, 1:100, 1:1,000, 1:10,000, is that which can be measured within 5 or 10 seconds after the liquid is poured into the watch-glass. It is very likely that there are at least two groups of substances which act powerfully upon the surface tension of the serum, and in different ways. The first group, in conformity with the substances already known, is adsorbed almost instantaneously in the surface layer, and determines the value of the surface tension of fresh serum and of serum solutions up to about 1:50,000, as given by the drop weight methods (with continuously renewed free surfaces); the other group which, on account of the techniques in use up to the present time, could not be detected is adsorbed in function of time, provided the free surface is left unstirred. This second substance, or group of substances, acts more powerfully than the other, and at extremely low concentrations. Indeed, we have seen that one-millionth part of serum is still active. Since serum does not usually contain as much as 10 per cent of solid substances in solution altogether, we may assume, and this is manifestly exaggerated, that the serum contains 5 per cent of these substances. Hence, this would mean that they are still active at such a low concentration as 1:20,000,000 (1 gm. in 20,000 liters, or over 5,200 gallons of water). The hypothesis of the two groups of sub-

⁶ du Noüy,¹ p. 579.

stances is expressed by Text-figs. 10 to 15, in which the initial value of the surface tension has been taken into consideration for the evaluation of the drop. By assuming that it is the same substance, or group of substances, which determines the initial value of the surface tension by very rapid adsorption, and the slow drop by slower adsorption, we obtain Text-fig. 16.

Hence, admitting the correctness of the assumption of the existence of two groups of substances, it becomes easy to account for the special grouping of the initial values of surface tension. Indeed, if we call the first group A, and the second group B, it is readily seen, by looking at the charts, that Group A, present at dilutions from 1:10 to 1:1,000 in sufficient quantities to have a marked action on the tension, is no longer effective in the case of high dilutions, from 1:100,000 up. There is a break in the initial value which is not always bridged by the 1:10,000 dilution. From 1:100,000 up, practically only Group B is active. On the other hand, we notice that it is precisely around the concentration of 1:10,000 that the drop is maximum. The reason is simple if we refer to Langmuir's ideas on the structure of the surface layer of solutions, when the solute is adsorbed in the surface layer. Langmuir⁷ has shown, indeed, that the free surface of such a liquid is generally composed of a monomolecular layer in which the molecules are geometrically disposed and similarly oriented with reference to a three dimensional space; in other words, that the surface of the liquid is composed of a sort of mosaic, each element of which is the same end of the same side of the molecules of the liquid. Furthermore, he states that the group molecules of organic liquids arrange themselves in such a way that their active portion is drawn inwards, leaving the least active portion of the molecule to form the surface layer. This hypothesis is supported by very striking facts, but their description would be beyond the scope of this paper.

Thus, in the case of serum solutions, the maximum drop would correspond to such a dilution as would allow the active molecules of Group B to dispose themselves with what is left of Group A in the most favorable way for the decrease of surface tension; and this is only possible at one precise concentration. At higher concentrations, the

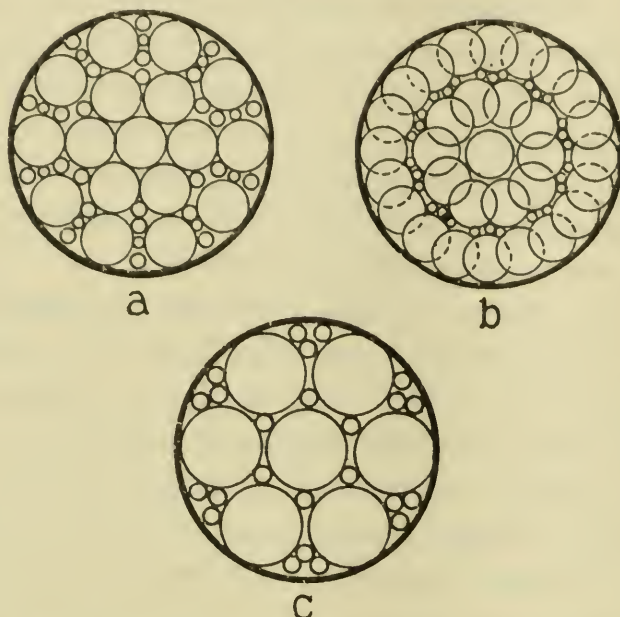
⁷ Langmuir, I., *Met. Chem. Eng.*, 1916, xv, 468; *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

excess of molecules of Group A interferes with the best possible geometrical arrangement of the molecules of Group B, and the forcing apart of these groups increases the surface tension. The same thing happens at higher dilutions, in which the forcing apart is due to the smaller number of molecules of Group B, which can no longer cover the liquid completely. This is, of course, a mere hypothesis which has, at least, the advantage of accounting for the facts found so far. But, whatever hypothesis is accepted, and in spite of the fact that the maximum drop is not so clear if the presence of only one group of substances acting together in the same way is assumed, there is no doubt but that an optimum concentration exists; it is easily explained, as well as the grouping of the initial values, on the basis of Langmuir's conception of the structure of liquid layers.

A glance at the charts will also show that the maximum drop does not always occur at the same concentration. Assuming the correctness of our hypothesis, this shift may be due to a change in the size of the group molecules. Indeed, the maximum drop occurs when the free surface is entirely covered with a certain lattice of molecules of Groups A and B, arranged in such a way as to produce the weakest force fields. Evidently there is an optimum concentration, as has been said before. But this optimum is function of the size of the molecules, and of the relative concentration of the two groups, A and B. This will be more clearly understood by looking at Text-fig. 27, *a* to *c*. In Text-fig. 27, *a*, the optimum is attained for a certain size of the molecules, and a certain relative number of both groups. The quantity adsorbed in the surface layer is exactly that which corresponds to the minimum surface tension. In Text-fig. 27, *b*, the size of the molecules is the same and the quantity adsorbed is greater, the concentration being higher. The arrangement of the molecule is no longer perfect, the proportion of Groups A and B is changed, and the molecules may be forced to tilt on one side, or to overlap. The result is that the surface tension is increased. In Text-fig. 27, *c*, we have another optimum arrangement, with a smaller number of larger molecules; that is, at a lower concentration. Although this illustration is very crude, it helps to visualize a plausible process through which the maximum may be shifted. As the molecules in solution are probably very long, with lateral branches, instead of

discs or spheres, as we have symbolically represented them, these figures must not be taken as an image of reality but as a mere comparison.

In the curves published, the maximum for human serum lies at 1:50,000, for the old dog serum at 1:30,000, for rabbit serum around 1:10,000, and for a very young pup at 1:7,000.



TEXT-FIG. 27, *a* to *c*. Hypothetical illustration of the possible arrangement of molecules in the surface layer of a serum solution.

IV.

Aspect of Crystallization of Serum Solutions.

When the solutions are allowed to evaporate, the sodium chloride crystallizes. By using watch-glasses, an interesting phenomenon is observed. When a pure sodium chloride solution crystallizes, it concentrates first, then the crystals are formed at the bottom of the glass as seen in Fig. 1. But when serum is added, very small crystals spread all over the watch-glass, and assume different aspects, according to the concentration, as shown by Figs. 2 and 3. Up to a concentration of 1:500,000 in the case of Serum 114, the effect of the lowering of the surface tension can be detected easily by the action of the substances adsorbed in the surface layer upon the molecules of sodium chloride. This phenomenon can be explained in the

following way. The large adsorbed molecules carry with them in the surface layer the Na and Cl ions. The concentration there becomes very high, and dissociation small. Sodium chloride molecules are formed and when the liquid evaporates, it progressively abandons the salt on the glass. No concentration occurs in the bulk, and no large crystals can grow at the bottom.

The phenomenon is similar when other surface-active substances are used, such as for instance saponin (Fig. 4).

This phenomenon is interesting because it shows that the presence of sodium glycocholate or taurocholate or dried serum in such a high dilution as 1:10,000,000 modifies profoundly the state of equilibrium and the distribution of phases in a liquid containing crystalloids in solution. Gibbs' statement that substances which increase the surface tension are more concentrated in the bulk is no longer true in this case. The birth of the membranes at interfaces, the first manifestation of the individuality of a cell, becomes very clear. The concentration of colloids and salts being much greater in the surface layer, precipitation will naturally occur there, and electric phenomena are forcibly different from those in the bulk. The aspect of the crystals is not the same at all dilutions, and at a concentration of 1:100, all sera studied so far have shown a very peculiar phenomenon, easily seen in the photographs. The more or less regular rings cannot be found as clearly at any other concentration. The white rings are very small sodium chloride crystals. The theoretical explanation of this phenomenon is not obvious. There is no doubt that it corresponds to different states of equilibrium of the surface layer, and possibly to different and periodical changes in its surface tension. This phenomenon is somewhat similar to that observed by Liesegang,⁸ obtained by adding 1 drop of a silver nitrate solution to a solution of gelatin and potassium dichromate.

As was to be expected, sodium oleate, glycocholate, and saponin act in the same way. Further investigations are being conducted on this subject.

⁸ Liesegang, R. E., *Z. Chem. u. Indust. Kolloide*, 1907-08, ii, 70.

V.

CONCLUSIONS.

The application of the ring method to the measurement of solutions of serum and of certain organic compounds has brought forth new facts, mainly the decrease of the surface tension of such solutions in function of time.

1. In serum diluted at such a low concentration as 1:1,000,000 in NaCl, physiological solution, the surface tension of the liquid is lowered by 3 or 4 dynes in 2 hours; at 1:100,000, by about 11 dynes (mean value) in 2 hours, and by 20 dynes in 24 hours; at 1:10,000 by about 13 to 16 dynes in 2 hours.

2. The drop in surface tension is much more rapid in the first 30 minutes and follows generally the law of adsorption in the surface layer in function of the time.

3. Stirring or shaking after the drop causes the surface tension to rise, but generally below its initial value.

4. The same phenomena occur when using sodium oleate, glycocholate, or saponin instead of serum.

5. For every serum, as well as for the substances mentioned above a maximum drop occurs in certain conditions at a given optimum concentration.

6. Not only are the substances which lower the surface tension adsorbed in the surface layer, in the case in which they are present with crystalloids, but also the crystalloids themselves, in contradiction to Gibbs' statement. This is plainly shown by the evaporation of such solutions in watch-glasses which, instead of a small group of sharp, large, well defined crystals at the bottom, leaves a white disc almost as large as the initial free surface itself, due to the liberation of the salt by the surface layer as it crawls down the concave surface of the glass.

7. In these conditions, solutions of serum are characterized by a very peculiar periodic and concentric distribution of the crystals, at a concentration of 1:100 only. The same ring-like aspect is observed with sodium oleate, glycocholate, and saponin, but not at the same concentration, as was to be expected, since serum is a solution in itself.

On page 735, Vol. xxxv, No. 5, Figs. 1, 2, 3, and 4, for *NaCl solution at 9 per cent* read *NaCl solution at 0.9 per cent*. On Plate 58 of the same article, at the lower left hand corner, for *NaCl 9 per cent* read *NaCl 0.9 per cent*.

EXPLANATION OF PLATES.

PLATE 58.

FIG. 1. Crystals of serum solutions in NaCl solution at 9 per cent. Serum 114 (rabbit).

PLATE 59.

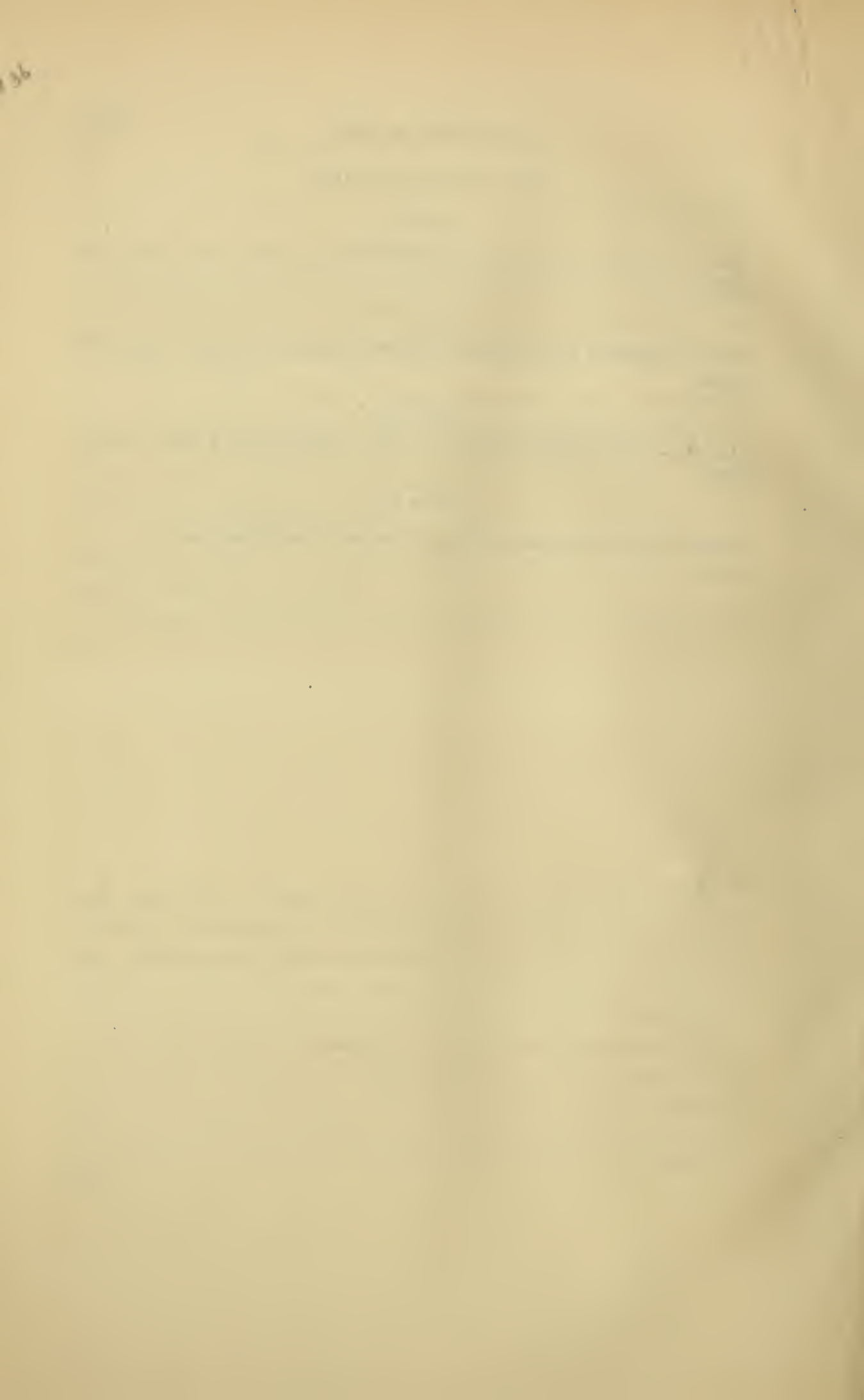
FIG. 2. Crystals of serum solutions in NaCl solution at 9 per cent. Serum 117 (rabbit).

PLATE 60.

FIG. 3. Crystals of serum solutions in NaCl solution at 9 per cent. Human serum.

PLATE 61.

FIG. 4. Crystals of saponin solutions in NaCl solution at 9 per cent.



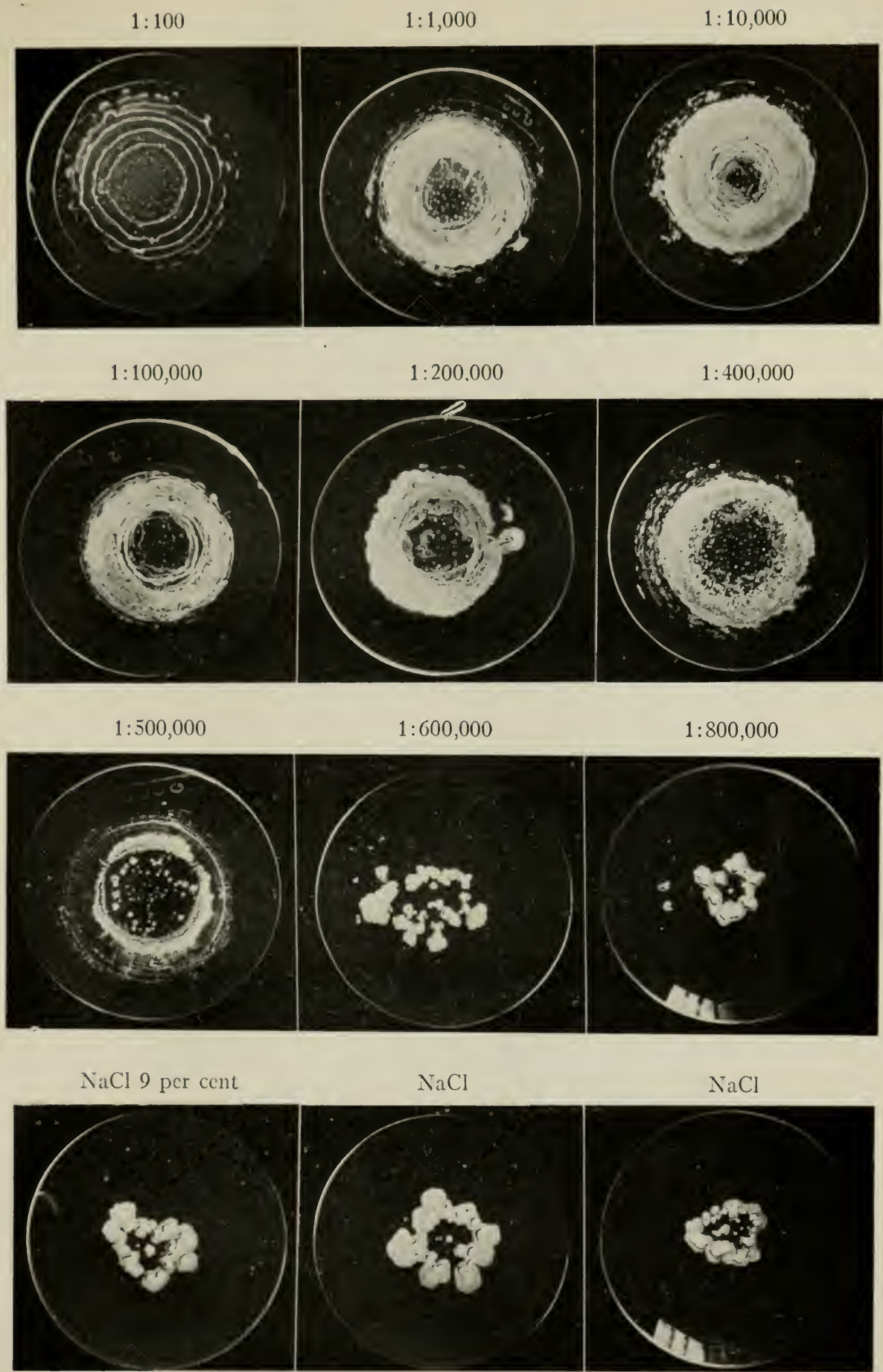


FIG. 1.

(du Nolly: Surface tension of serum. II.)

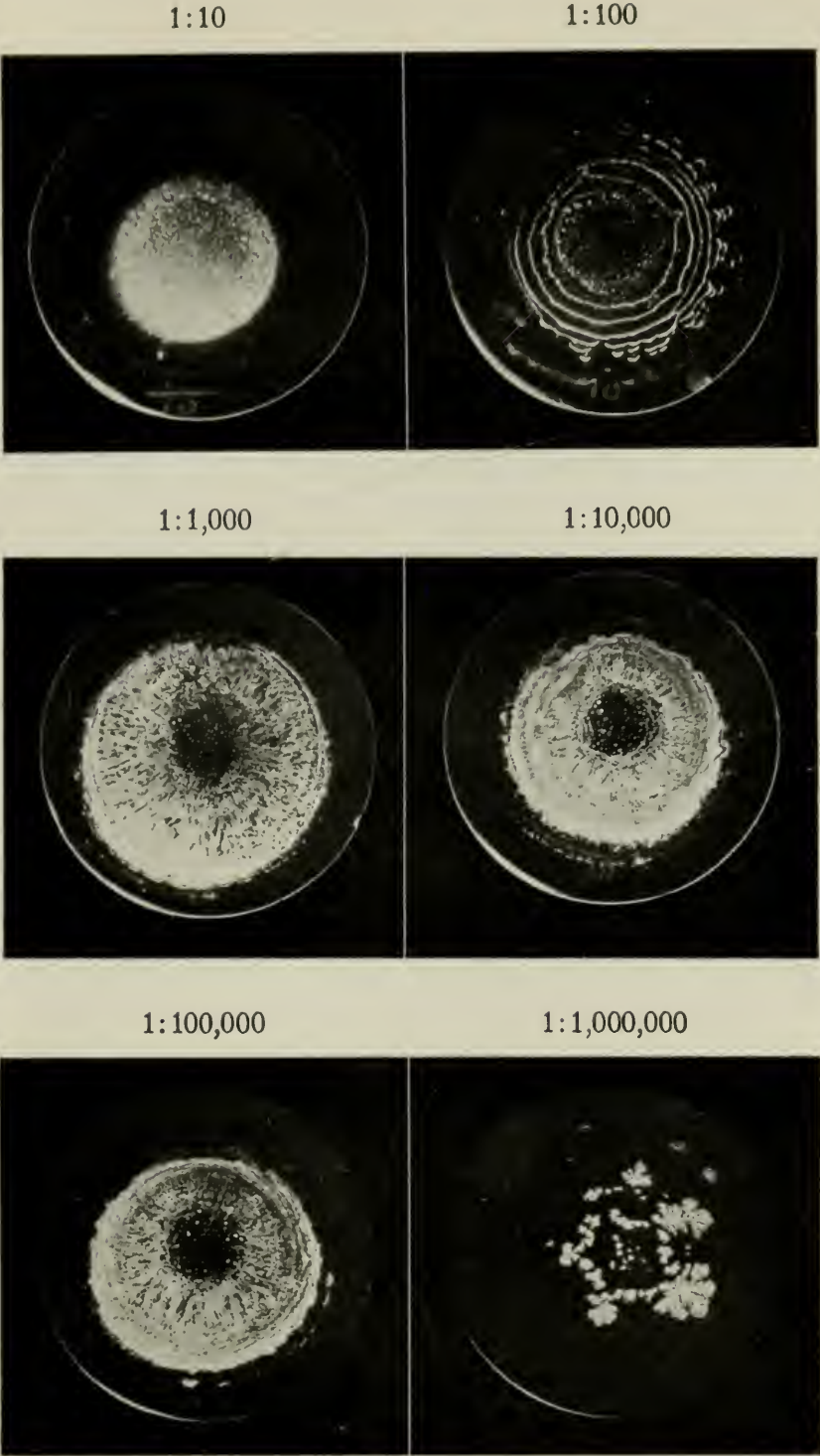


FIG. 2.

(du Noüy: Surface tension of serum. II.)

736^a

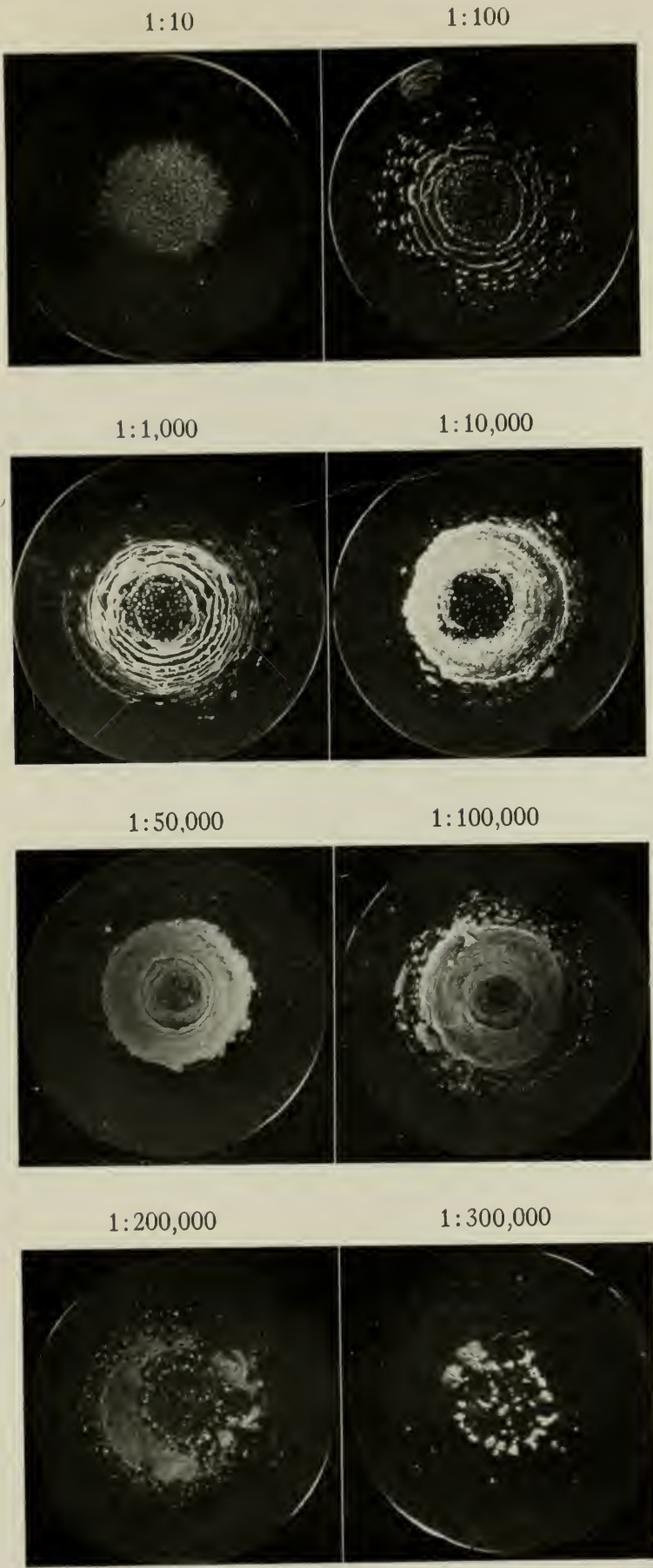


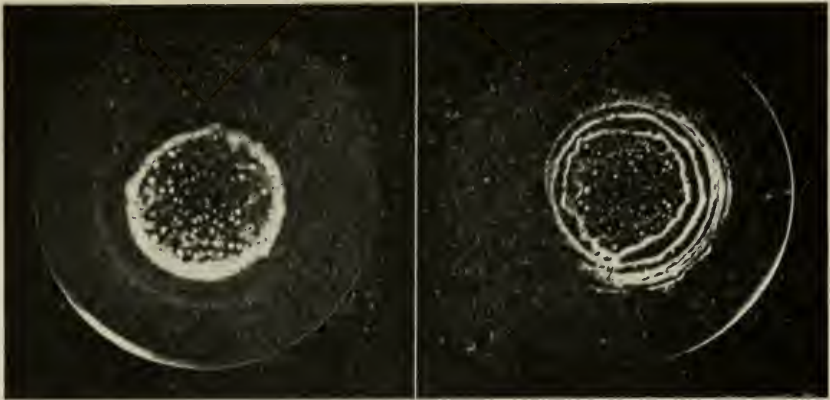
FIG. 3.

(du Noüy: Surface tension of serum. II.)

6362

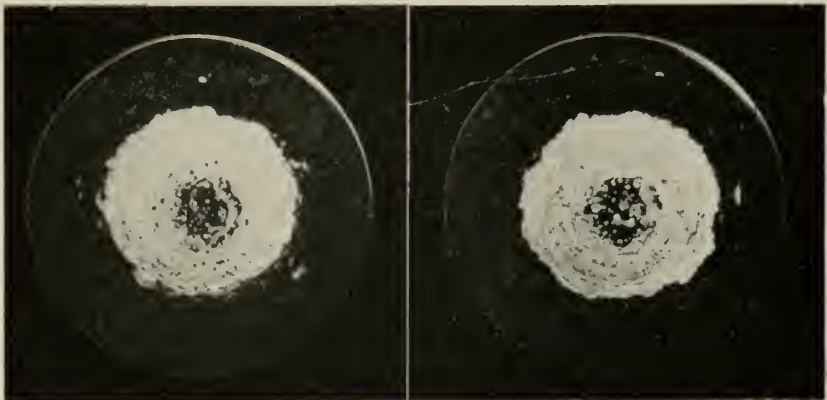
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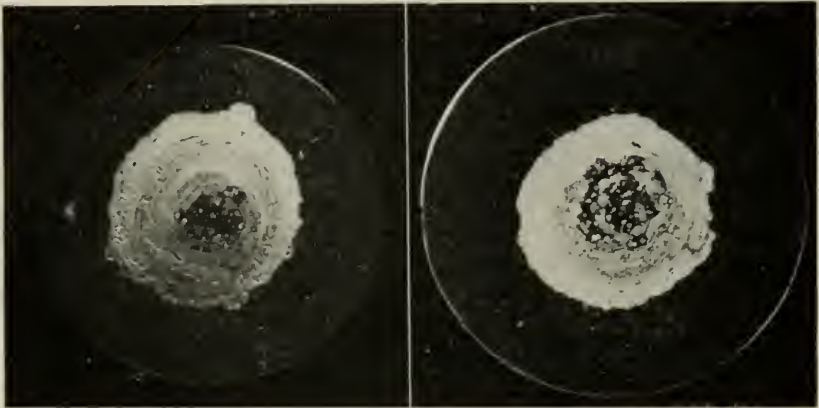
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1:1,000,000

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NaCl

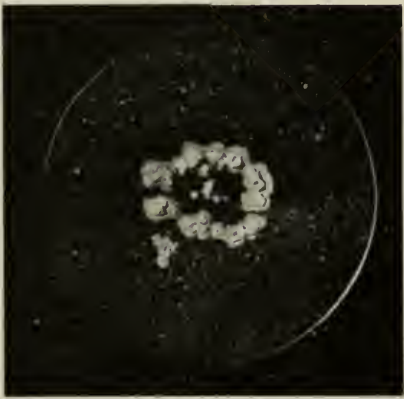


FIG. 4.

(du Nolly: Surface tension of serum. II.)



PATHOLOGY OF THE DERMATITIS CAUSED BY MEGALOPYGE OPERCULARIS, A TEXAN CATERPILLAR.*

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PLATES 62 AND 63.

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INTRODUCTION.

A small larval moth has been causing dermatitis in southern Texas for the past decade, particularly during the years 1913 and 1920. It is known locally as the puss-caterpillar, opossum bug, Italian asp, and, by Mexicans, *el perrito* (the puppy), and belongs to the family Megalopygidæ, of the group known as slug caterpillars. As it has not been investigated systematically from a medical standpoint up to the present time, the work reported in this paper was undertaken at the suggestion of Mr. F. C. Bishopp, of the United States Bureau of Entomology, who is stationed at Dallas, Texas, and to whom the writer is much indebted for the caterpillars studied and notes concerning their distribution and injurious effects.

Mr. Bishopp reports the following clinical symptoms from regions infested by the caterpillars. All stages of the larva are capable of inflicting stings, the intensity of the lesions being in proportion to the size of the caterpillar. There is a marked variability in the susceptibility of different individuals. At some seasons there have been epidemics of dermatitis so widespread that public schools in San Antonio, Texas, were closed until the surrounding trees could be sprayed.

The severity of the sting varies with its location and the thickness of the skin affected. There is at first a localized, painful area of erythema, which burns intensely. Small vesicles then appear; whitish

* An abstract of this article was read before the Research Club of The Harvard Medical School, December 16, 1921.

spots on the red background and slight swelling may be present in the vicinity of the lesion. Sometimes a sting upon the wrist will cause a swelling of the entire arm. There may be generalized symptoms of numbness in the part affected, or even in other limbs, with a pseudo-paralysis. Carpenters have reported that they were practically unable to grasp a hammer or to work for several hours after being stung upon the wrist or hand. In children there may be from 1-5° of fever, extreme restlessness, and often nausea and vomiting, mild general spasms sometimes following the attack. As the local symptoms subside the skin may remain discolored for some time, reddish or almost blackish areas marking the site of the lesion.

Megalopyge opercularis.

The caterpillar is the larval form of the moth *Megalopyge opercularis* (Fig. 1), which has a distinctly southern distribution in this country, occurring abundantly only south of the Red River in Texas, although it is found casually in the Southern States. Other species of the genus are found in Mexico, Central and South America (thirteen species in Brazil (von Ihering, 1914)), and the West Indies. It is a comparatively small larva, measuring about 2 cm. in length and 1 cm. in width, if one counts in the soft, luxuriant coat of tawny to grayish hairs which gives it its popular names. Its color varies from dark fawn, through tawny red, to gray, and the caterpillars vary in size, some being as large as the drawing in Fig. 1, others almost a third smaller. Beneath the hairs are concealed the tubercular ridges, or verrucæ, common to many caterpillars, which bear rows of sharp, needle-like spines (modified setæ) like porcupine quills, which are hollow structures that penetrate the human skin and cause the dermatitis just mentioned. In general appearance the caterpillar so closely resembles the larva of *Lagoa crispata*, the flannel-moth, which occurs along the Atlantic Seaboard to New England and the Northern States, that the two species are practically indistinguishable to an inexperienced observer. For a detailed description of the adult moth of *Megalopyge opercularis*, of the various instars of the larva, of its cocoon, and of its habits, the reader is referred to an article by Mr. Bishopp, which is to be published shortly.

The dorsal setæ, or hairs, of *Megalopyge opercularis* are twice as long over the ninth and tenth abdominal segments as they are over the first eight, and form a fairly conspicuous tail. The fourth and fifth ocelli are close together in *Lagoa*, rather distant from each other in *Megalopyge*.

The family Megalopygidae belongs to the suborder Frenatae and comprises three genera: *Carama*, *Lagoa*, and *Megalopyge*. It is important from an entomological standpoint as forming one of the links in the Zygænoidea, standing between the Zygænidae and the Cochlidiidae (or Limacodidae). "Its members are particularly interesting as transition forms and their structure is the clue to the peculiarities of the slug caterpillars" (Fracker, 1915). The distinguishing features of these genera are fully described by Fracker. All of their North American species are capable of inflicting more or less painful stings through the agency of poisonous spines, the anatomy of which will be described in the course of this paper.

Urticating Caterpillars.

Three superfamilies of moths include practically all of the urticating caterpillars, the Zygænoidea, Bombycoidea, and Saturnoidea. In Table I the principal families are grouped under these superfamilies and a few representative genera are noted under each, with their common names. The list is by no means complete; it is meant as a suggestive tabulation, rather than a classification. Fracker's classification has been followed, which accounts for the presence of *Automeris io* under the Hemileucidae, rather than under the Saturniidae (Table I).

Geographical Distribution.—Urticating caterpillars are world-wide in their distribution, but the most offensive species appear to inhabit South America.

Europe.—While all three superfamilies are represented here, two genera stand out as being the most troublesome, the processionary caterpillars of the genus *Thaumetopæa* (*Cnethocampa*) and the brown-tailed moth, *Euproctis chrysorrhæa*, well known in the Eastern States of this country. The first named genus has been known from antiquity, the ancients employing it as a vesicant. Lists of the European urticating caterpillars will be found in Tyzzer's article (1907) on brown-tailed moth dermatitis and in Railliet's text-book (1895).

Asia.—Caterpillars of the family formerly called Bombycidae have been known to cause trouble in Ceylon and India (Castellani and Chalmers, 1913), and Tyzzer mentions a Philippine caterpillar, *Taragama igniflua*, whose spines are somewhat similar to those of the Megalopygidae, but slightly barbed, instead of smooth shafted.

Africa.—Four families are mentioned in Castellani and Chalmers' text-book as being capable of producing urtication: (1) the larva of a tiger-moth, of the

TABLE I.

Superfamily.	Family.	Genus.	Species.	Common name.	Habitat.
Zygænoidea.	Megalopygidae.	<i>Carama</i>	<i>cretata</i> .		United States.
		<i>Lagoa</i>	<i>crispata</i> .	Flannel-moth.	" (Northern).
		<i>Megalopyge</i>	<i>opercularis</i> .	Puss-caterpillar, etc.	" (Southern).
		"	<i>lanata</i> .	<i>Táta-rána</i> , <i>gusano pelo de Índio</i> .	Brazil, Colombia, etc.
		"	<i>albicollis</i> .	<i>Táta-rána</i> .	"
Bombycoidea.	Cochliidiæ. (Limacodidae).	"	<i>radiata</i> , etc.	" <i>pollo</i> , <i>perrito</i> .	" Colombia, etc.
		<i>Sibine</i>	<i>stimulea</i> .	Saddle-back moth.	United States.
		<i>Natala</i>	<i>nasoni</i> .		"
		(Various Brazilian genera.)			South America.
		<i>Thaumelopæa</i>	<i>processionea</i> .	Processionary caterpillars.	Europe.
		(<i>Cnelhocampa</i>)	<i>pityocampa</i> .		
			<i>pinivora</i> .		
		<i>Taragama</i>	<i>igniflua</i> .		Philippines.
		<i>Euproctis</i>	<i>chrysorrhæa</i> .	Brown-tailed moth.	Europe, United States.
		<i>Arctia</i>	<i>carya</i> .	Woolly bear.	" Canada.
Saturnoidea.	Lasiocampidae. Liparidæ. Arctiidae.	(Unclassified?)		<i>Oruga Santa Maria</i> .	Colombia.
		(Various Brazilian forms.)			South America.
		<i>Hemileuca</i>	<i>maia</i> .	Buck-moth.	United States.
	Saturniidae. Hemileucidae.	<i>Automeris</i>	<i>io</i> .	Io moth.	"
		"	<i>cinctistriga</i> .	<i>Gusano perejil</i> .	Colombia, South America.
		"	<i>viridescens</i> .		Brazil, "
					"

Arctiidae; (2) a member of the Cochlidiidae; (3) one of the Liparidae (to which the browntail belongs); and (4) a member of the old group Bombycidae, now subdivided into several new families.

North America.—The best known and most troublesome caterpillar on this continent is the larva of *Euproctis chrysorrhæa*, the brown-tailed moth. The pathology of the lesions it produces, as well as the mechanism by which they are brought about, were studied and reported by Tyzzer in 1907. A list of the native caterpillars that have urticating hairs will be found in Herrick's (1916) and Riley and Johannsen's (1915) text-books, and will be seen to contain members of all three superfamilies previously cited. Four of the more commonly encountered native caterpillars with urticating propensities are: *Lagoa crispata*, the flannel-moth, studied and reported by Packard (1894); *Hemileuca maia*, the buck-moth; *Sibine stimulea*, the saddleback; and *Automeris io*, the Io moth. The last is much sought after on account of the beauty of its imago, and is probably the best known of the four.

South America.—This continent, particularly its northern portion, is the habitat of many noxious caterpillars, belonging to the three superfamilies mentioned above. Roughly speaking, there are five groups of stinging caterpillars in that region: (1) those belonging to the family Megalopygidae (thirteen species), represented by the popular group *táta-rána* of Brazil (Göldi, 1913; von Ihering, 1914; Bleyer, 1909); (2) those included in the Cochlidiidae and occurring in Brazil; (3) those of the family Arctiidae, found in Brazil and Colombia (*oruga Santa Maria* (Garcia, 1910)); (4) a group resembling our buck-moth and Io moth, and belonging to the Hemileucidae, found in the same general regions (Göldi, Bleyer, and Garcia); and (5) a group belonging to the Saturniidae and described by Bleyer in Brazil. These varieties are illustrated and described in the articles just cited. By far the best article on the urticating caterpillars of Brazil is that of von Ihering (1914), to which the reader is referred for further particulars concerning the *táta-ránas*. This name is from the Tupi-Guarani dialect and simply means fire-like; it is thus popularly applied to any urticating caterpillar, although it usually means one of the Megalopygidae, the true *táta-ránas*.

The distribution of the stinging caterpillars has been outlined here at some length, because I have not been able to find it summed up in any one article thus far written.

Lesions and Symptoms Caused by These Genera.—The lesions produced by the sting of these genera vary from simple erythema, with burning or itching, to more extensive inflammation, with the production of papules, vesicles, or bullæ. The conjunctiva is sometimes affected by nettling hairs that retain their irritating properties after becoming detached from the caterpillars, as in the case of the processionary and brown-tailed moths. Cases of inflammation of the respiratory mucosa have been reported in connection with the former (Laudon, 1891). Goossens (1881, 1886) experimented upon himself with this caterpillar and produced very unpleasant symptoms, his entire body becoming swollen and his eyelids so edematous that he could no longer see to continue the experiment. Cases of like

gravity are described by Göldi, Bleyer, von Ihering, and Garcia, most of them occurring in connection with members of the genus *Megalopyge*. Their sting causes intense pain, swelling of an entire member, lymphadenitis, and stiffness and disability of the affected side. Bleyer describes a sphingid moth whose sting produces twitching of the affected member and even of the facial muscles on the affected side. Garcia describes symptoms of conjunctivitis and tonsillar angina following the sting of *Megalopyge lanata* (*gusano pelo de Indio*), "severe enough to be considered potentially fatal."

Types of Nettling Hairs.—There are two large classes of nettling hairs, those readily detachable and those that do not come off easily, but break off in the epidermis of the victim after direct contact with the insect. The latter type may be simple and quill-like, or with barbs (*Taragama*); or they may be branched like small trees. Various types are shown in Fig. 6, from Göldi's article. The detachable type is represented by the nettling hair of the brown-tailed moth and processionary caterpillars, which is dangerous after an entire season, following detachment from the insect. The smooth, spinous type with which we are concerned in this paper is described by Packard (1894, 1898) in connection with *Lagoa crispata*. He believed the venomous fluid to be contained in the spines and to be secreted by several large cells at their base. Von Ihering (1914) describes an almost identical apparatus for an unspecified species of *Megalopyge* which he studied in São Paulo, Brazil. Leydig describes "poison" oozing through a pore in the tip of such a seta, but Berlese thinks this erroneous and believes that the venom leaves the shaft by osmosis through the chitinous wall. Discussing this question, von Ihering says that he has thought on several occasions that he could demonstrate a subapical pore in these spines, similar to that in a serpent's fang. He also says: "Upon irritating the animal, it erected its hairs in the usual manner and we then saw a little drop of liquid exude from the tip of each of the spines which serve to defend the caterpillar." He goes on to point out that, should the spine be broken off in the skin of the victim, it would be useless for further action. As will be seen later this is not necessarily true, for the venom dries and forms a pigmented, gummy plug that effectually seals the broken extremity.

Nature of the Venom.—As to the nature of the poisonous substance, very little is known. Goossens believed that he had isolated cantharidin from the hairs of the processionary caterpillar; but Laudon disproved this, substituting the conjecture that it is formic acid, a supposition that has held with considerable tenacity up to the present, although it is obvious that this acid alone cannot cause the symptoms produced by the sting of these caterpillars. Bleyer noted a strong odor of this acid in broken spines from a sphingid caterpillar but tests with litmus proved the substance to be alkaline. Tyzzer experimented upon the solubility of the venom of the browntail caterpillar, as well as with its reaction to heat, chemicals, etc., but came to no definite conclusion as to its chemical composition. He found that it had a tendency to break up the rouleaux of human erythrocytes and to shrink the latter after a brief phase of crenation. The venom is present in the spines in such minute quantities that an analysis is extremely difficult.

Original Work on Megalopyge opercularis.

Production of Gross Lesions.—The larvæ used in these experiments were shipped from Texas in mailing-tubes; some of them, however, had spun cocoons in transit and were used in their prepupal form. The larva was rubbed over the slightly moistened skin of the bend of the elbow, anteriorly in four individuals. The prickling of the tiny spines was distinctly felt in each case. Next a burning sensation was experienced, which began almost immediately in the individuals with thinner skin, and appeared a minute or-so later in those with thicker skin. This persisted from $\frac{1}{2}$ hour in one case to 3 to 5 hours in the others. Shortly after the onset of burning, several small papules about 2 mm. in diameter, with a yellowish red cast, appeared over the surface of the erythematous area. The latter attained a diameter of about 4 cm., but the concentric rings of red and white, described by Mr. Bishopp as encircling it, were not observed in any of these four experimental cases. After the erythema subsided the papules persisted for about 48 hours to 3 days; they formed no large vesicles and itched in only one case out of the four. There was a suggestion of slight hemorrhage in their vicinity, giving them a petechial appearance.

A second application, 2 days later, in the case of the writer, produced more marked symptoms and slight itching. A third, about 5 days after the first, was carried out on both forearms in multiple, several caterpillars being tested out for other phases of the experiment. In this case the reaction was somewhat different; the erythema was less painful, but no less intense, and was followed by the production of wheals of an average diameter of about 2 cm., which itched intensely. These had small yellowish, slightly vesicular papules superimposed upon them and persisted for 3 days, after which the itching and swelling gradually subsided. Small reddish papules, with tiny scabs at their apices, were still present after a week. One of the lesions was excised for examination at the height of the inflammation, after 24 hours development, by plunging a skin hook into its center and clipping off an ellipse of skin 1 by 0.5 cm. in size, with straight scissors. This was done without anesthesia, in order to avoid distorting the lesion. The wound healed uneventfully, the caterpillar venom causing no complications. During the develop-

ment of the multiple wheals the forearm became hot, itched intolerably, and there was a sensation of dull pain extending distally to the wrist; but no paralytic or generalized symptoms were noted. The prepupal stage of the larva caused similar, but slightly less intense symptoms, whereas a dried, dead larva was only urticating when vigorously rubbed in. Apparently the toxicity diminishes somewhat after the death of the caterpillar.

Although it was almost certain that the setæ, or spines of the tubercles, were the cause of the reaction, the other hairs, which are long and feathery, were cut off and rubbed into the moistened skin; merely a slight scratching was produced. Juices of the skin and body cavity of the caterpillars were inoculated into small wounds made by cross-hatching the skin with a sterile sewing needle. Although the juices of the brown-tailed caterpillar were found to be very, and almost immediately toxic by Tyzzer, those of *Megalopyge opercularis* produced no reaction. This coincides with the findings of von Ihering, who experimented with inoculated body fluid of a *Megalopyge* and produced no lesion, although the venom from its setæ promptly caused a typical reaction.

Next a verruca was cut off the caterpillar's skin and held in fine forceps so that its setæ were presented to the skin of the subject like the bristles of a brush. Brisk rubbing gave results precisely like those obtained by rubbing the entire larva over the integument; the venom is, therefore, contained in these spines and limited to the verrucæ. In order to test for the presence of venom in the poison sacs and hypodermal glands (which will be described later), a caterpillar was pinned down on a board and eviscerated. The inner surface of the skin was sponged clean with cotton, scraped with a scalpel and the scrapings were rubbed into scarified areas on the human skin. Then, after scraping as much as possible, without tearing, a small square of the caterpillar's skin was excised and placed with its inner surface closely apposed to a second scarified area on the writer's arm. Both experiments produced positive results, although a control scarification showed no such change. Setæ pulled out and held base downward were rubbed into an excoriated area; this also produced very slight, but positive reactions. These three experiments show that the venom is contained in the cuticle of the caterpillar and in the base of the setæ as well as in the hollow shaft.

Rabbits' ears rubbed over the inner surface with the larvæ become reddened and swollen and then droop for about 24 hours; but they show no wheals, vesicles, or papules. A caterpillar rubbed over the shaven abdomen of a mouse produces almost immediate symptoms, the mouse scratching and licking the inoculated area for about $\frac{1}{2}$ hour. Tiny vesicles and a skin reaction similar to that seen in the human subject were apparent; under the microscope the lesions showed a simple leucocytic infiltration.

Treatment.—The dermatitis responds rather readily to any soothing lotion or antipruritic; the proprietary Obtundia Cream was found especially soothing, although carbolated vaseline was also effective. If the lesions become excoriated, compound tincture of benzoin or tincture of iodine is indicated. The immediate application of an alkali, such as ammonia, is without any beneficial effect and is not to be recommended; in spite of it the erythema and vesicle formation proceed unabated.

Anatomy of the Urticating Apparatus.—The anatomy of the stinging apparatus is comparatively simple. A number of transverse tubercular ridges, or thickenings, are distributed over the surface of the cuticle of each segment. Roughly speaking they are laid down in six parallel longitudinal rows over the dorsum and sides of the various segments. These are the verrucæ, whose arrangement, as well as that of the setæ they bear, is utilized by entomologists in classifying the insects. The exact arrangement of these verrucæ and setæ is given by Fracker, The tubercles consist of weakly chitinized thickenings of the cuticle, upon which are rows of bristle-like spines and tufts of feathery hairs. The former are firmly, the latter rather lightly attached, so that if the larvæ be scraped, these hairs, rather than the spines, come away. The latter are best seen in the prepupal form of the moth, for the hairs having been utilized in spinning its cocoon, the insect is comparatively naked, the verrucæ standing out clearly as bristling ridges (right hand drawing in Fig. 1). They have the appearance of minute pincushions, the spines radiating somewhat from their convexity; their grayish color and blackish tips stand out conspicuously.

If a verruca be excised and examined under the microscope (Figs. 2 and 3), it will be found that these setæ are slender, acute at one end, and attached to a conical or rounded projection of the verruca by a cup-shaped expansion at the base. They are of a yellowish brown color, hollow throughout, and contain masses of amorphous, dark brown pigment scattered through their lumina. They are frequently

found with broken tips, in which case a plug of pigment projects slightly from the fractured extremity. They vary in size from 0.3 to 1.0 mm. in length and from 15 to 45 μ in diameter. Apparently most of them are broken at the tips, but the smaller forms are found to taper to a needle-point that does not seem to be canalized, although the lumen runs down very nearly to the apex. Tests show them to be composed of heavily chitinized material, the pigment being resistant to solvents.

Microscopic Anatomy.—Larvæ and prepupal larvæ were fixed in Zenker's fluid, cut to 5 μ in paraffin, and stained with hematoxylin-eosin, eosin-methylene blue, and phosphotungstic acid-hematoxylin. Microscopic examination shows that the cuticle is thickened to form the verrucæ (Fig. 2) and, instead of being solid and laminated as elsewhere, is filled with cells resembling those of the hypodermal layer. In fixed material these cells shrink away from the cuticle, lying in globular cavities; but unfixed material does not show any unfilled spaces. As the surface is approached one finds the cells increasing in numbers until, in the upper layers, they almost touch one another. The surface layer shows poison setæ and ornamental hairs alternating with one another. Beneath the verrucæ there is a heaping up of the hypodermal layer of cells to form gland-like organs, whose ducts spiral outward somewhat like those of the human sweat glands and communicate with the hair roots. As these readily can be demonstrated to connect with the roots of the feathery hairs, but only occasionally are seen to lead into that of a poison seta, it is questionable whether they are still functioning in this case; they may have transmitted the venom to the poison sac of the seta at an earlier data and then atrophied or they may be columns of setigerous cells, as interpreted by Packard (1894) in connection with *Lagoa crispata*. According to von Ihering's interpretation, they are at first trichogenous, forming the seta, and then become toxogenous, secreting the poison that is stored in the theca, or poison sac. As they appear to be atrophic in the stage examined here, although the setæ are highly venomous, this point will have to be decided by a careful study of the setæ in various instars of the caterpillar.

The structure of the poison setæ is best seen in the smaller and medium sized forms of the larger tubercles. Fig. 4 shows one of these

diagrammatically, the drawing being a reconstruction from serial sections. The shaft (*Seta*) is hollow and broken off at its tip, which is plugged with a mass of pigment (*Pigm.*) The base expands into a chitinous collar, continuous with the shaft, which embraces a conical projection of the cuticle (*Coll.*), forming an inner collar. Just above this is a chitinous diaphragm (*Diaphr.*) pierced by a small opening, or fenestra. The cuticle forms a shell (*Bulb*) for the internal structures of the organ and usually shows one or two nuclei at its base (*Bulb n.*), which may belong either to the setal root, or to the theca, or poison sac, though the latter seems unlikely, as they differ somewhat in appearance from those of the sac. At the base of the cuticular shell is a carrot-shaped structure, staining darkly with phosphotungstic acid-hematoxylin and with eosin, which appears to connect with the remnant of one of the spiral ducts or cell columns in some instances. This is not universally present and is found in a small proportion of the setal bulbs. It is similar to the root of the feathery hairs in its appearance, situation, and staining properties, and is possibly a vestigial root (*V. r.*).

The theca, or poison sac, so called because of its anatomical continuity with the duct that traverses the setal shaft, although it cannot yet be demonstrated that its contents are nothing but the venom, is a thin walled bulb with several delicate, flattened, elliptical nuclei sparingly distributed over its surface (Fig. 4, *P. s.*; Fig. 5). It communicates with a slender tube, or duct (*P. d.*), constricted as it traverses the fenestra in the diaphragm and expanding slightly to terminate somewhere near the tip of the shaft, where it thins out and becomes difficult to follow. Nuclei are scattered over its surface and are best seen in phosphotungstic acid-hematoxylin sections. No muscular tissue is demonstrable in connection with the sac or duct. Both of them are filled with a continuous mass of finely granular, fixable substance in which bubble-like vacuoles are seen occasionally. The wall of the sac, although it contains several nuclei, shows no cell partitions in the sections examined; it appears to be syncytial, though silver nitrate preparations might demonstrate cell boundaries. Sometimes a delicate membranous partition appears to bisect the sac in a plane at right angles to its long axis.

These basal organs of the setæ vary somewhat in respect to shape and location; they may be as represented in the figures, or constricted at their middle to form a figure of eight. They may lie embedded in the cuticle, with the collar of the shaft flush with the surface, or they may project so far above the surrounding cuticle as to be constricted at their base to a degree that renders them pedunculated. Such setæ are more readily detached than their more deep seated neighbors.

The poison apparatus of this caterpillar corresponds in every particular with that of *Lagoa crispata* as described by Packard (1894, 1898) and of the *Megalopyge* described by von Ihering (1914), which is to be expected in the case of such closely allied genera; it is totally different from the unicellular apparatus of the genera described by Holmgren.

Pathology of the Lesions in Human Skin.—The lesion excised from the arm was dropped immediately into Zenker's fluid and, after fixation, cut into some 280 practically serial sections at $7.5\ \mu$. They were stained in eosin-methylene blue and phosphotungstic acid-hematoxylin. There are approximately ten fragments of setæ found embedded in the epidermis of the lesion; none of them penetrates to the corium, and yet the latter shows marked pathological changes. The seta enters the epidermis by pushing between cells and forcing them into a compact, encircling layer in its immediate vicinity (Figs. 7 and 8). Where the setal shaft is intact this is the only effect. Some of the setal fragments represent the tip broken off in the epidermis, others portions of the shaft that apparently have lost their tips before penetrating the skin, like the extremity of that in Fig. 4. Where the lumen of the shaft communicates with the surrounding epidermis, the cells of the latter are killed or dissolved (Figs. 7 and 8). A coagulum similar to that found in the venom sac and seta of the insect can be seen in the shaft fragment, oozing out into the cavity left by the destruction of the epithelium. Sometimes the necrosed epithelium forms a denser and more darkly stained coagulum. The loss of cells about the setal fragment is comparatively extensive, and vesicular cysts constitute the typical lesion (Figs. 9 and 10). These lie in the epidermis and may or may not show setal fragments in their walls, according to the section. They usually contain short threads of fibrin in a clear space and are invaded by a moderate number

of lymphocytes in their more advanced stages. They measure from 150 to 350 μ in diameter, apparently varying directly in proportion to the size of the seta that caused them. The usual diameter is 150 μ and is remarkably constant. The largest lesion (Fig. 10) was found to lie beneath a large puncture wound, shown in the photograph, filled with coagulum which showed small vacuoles. The puncture measures 30 μ in diameter, the size of one of the larger setæ; the latter may have entered the skin and been withdrawn, leaving its venom behind, for no remnants of the seta can be found. The average diameter of the setal fragments found in the skin is from 14 to 18 μ , therefore the ratio of the diameter of the lesions caused by the smaller setæ to that of those caused by the larger is approximately the same as the ratio between the setal diameters.

Above the vesicles the keratinized layer of the epidermis is raised to form a small blister, filled with granular coagulum, but this is not always the case. Occasionally a small scab of coagulated and necrosed epidermis surmounts them; this is the tiny scab that persists after the lesions have subsided. Below the vesicular, primary epidermal lesion, the cells of the prickle and germinative layers are separated and degenerated and the tissue is edematous. Almost every blood vessel in the edematous corium is surrounded by an exudate of lymphocytes, eosinophils, and endothelial and polymorphonuclear leucocytes (Fig. 11). The lymphocytes outnumber the other cells very noticeably, the eosinophils are next in order of frequency, and the endothelial and polymorphonuclear leucocytes about equally represented and not very numerous. The lumina of the capillaries show many lymphocytes and eosinophils, and those of the lymphatic system are sometimes almost clogged with the former. The vascular endothelium is not appreciably swollen, nor are its cells increased in number, no mitoses being found in them.

The reaction is, therefore, of the type that Tyzzer describes in connection with the brown-tailed moth; the similarity is at once apparent if one compare the illustrations of this article with his. It is a deeper and more destructive lesion than is that of the browntail and is essentially what one would interpret as a reaction intended to neutralize a poison, as lymphocytes are the most abundant cells in the perivascular exudates. Eosinophils, so commonly seen in skin reac-

tions in connection with cellular decomposition, are second in order of frequency. These leucocytes also respond to the secretions of animal parasites and to vegetable proteins (*cf.* asthma and hay fever); the character of the reaction, therefore, points to a protein irritant.

Nature of the Poison of Megalopyge opercularis.—Boiling a caterpillar in water for a few minutes, after killing it, renders its venom inert; after drying, the spines may be rubbed into the skin with impunity. An hour in water at 55°C. has no effect upon the venom, but this temperature will have the same effect as boiling if the caterpillar be left in water at 55° over night. It is impossible to extract enough venom from the setæ of a few caterpillars in a little water or salt solution to get any definite chemical reactions. Such extracts are also unsatisfactory for testing out on the skin.

On the other hand, it is possible to obtain the venom in another way. By extracting the ground skin and hairs of these larvæ and their prepupal form with distilled water, or salt solution, an impure mixture of extractives is obtained. This contains the venom and causes symptoms when inoculated into the human skin, or injected into animals. A detailed description of the work done in this way will be reserved for another paper. It will be enough to state here that these extracts cause the same reactions in the human skin (burning, erythema, and itching, with the production of wheals and vesicles) as do the caterpillars themselves. Injected intravenously into rabbits they produce pupillary contraction, conjunctival edema, restlessness, and convulsions and death when administered in sufficiently large doses. Given intraperitoneally to mice they cause roughening of the coat, profuse defecation, with retraction of the flanks, stiffened and uncertain gait, and death in an hour following the administration of 10 mg. of crude desiccated extract in 0.5 cc. of normal salt solution. When applied in small quantities to isolated strips of cat smooth muscle *in vitro*, they intensify the rhythmic contractions and slow down their rate. This effect is almost immediate and produces exhaustion and death of the strip after 15 minutes or so.

CONCLUSIONS.

1. The dermatitis caused by contact with the larva of *Megalopyge opercularis* is produced by a poison introduced by the hollow,

specialized setæ of its cuticular tubercles. It is not produced by the ornamental hairs, or by the tissue juices of the animal.

2. The poison appears to be of the nature of a venom, combined with protein vehicles, and may be itself a protein.

3. It is rendered inert by boiling, or by heating to 55°C. for a considerable period of time.

4. It is apparently stored in sacs at the base of the setæ, but whether secreted there, or by hypodermal glands, remains to be determined.

5. It diminishes in virulence after the larva has spun its cocoon, and is no longer active after the caterpillar is dead.

6. The poisonous spines cause localized necrosis of the human epidermis, followed by the formation of small vesicles. The cellular reaction to the poison is chiefly lymphocytic.

The writer is very grateful to Mr. F. C. Bishopp, of the United States Bureau of Entomology, for the opportunity of studying this caterpillar, for the caterpillars furnished, and for his advice and information. So many individuals from the Bussey Institution, the Smithsonian Institution, and various departments of the Harvard Medical School have given me advice and help that it is impracticable to list their names; their assistance is none the less appreciated.

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EXPLANATION OF PLATES.

PLATE 62.

FIG. 1. Pencil drawing of the extended and contracted larva of *Megalopyge opercularis* and of the prepupal stage of the caterpillar (head downwards). Note the rows of blackish setæ in the prepupal phase and the comparative absence of long hairs. Slightly enlarged.

FIG. 2. Section of a verruca. This shows the thick cuticle with a row of setal bulbs on its surface, two of them with setæ attached. Note the carrot-like root of an ornamental hair near the middle of the row, with its spiral-shaped lower extremity. The barbed hairs are the non-stinging, ornamental type. In the body of the verruca is the gland-like thickening of the hypodermis, which may be connected with the secretion of venom. Eosin-methylene blue. $\times 100$.

FIG. 3. Unstained setæ attached to a verruca which has been cut off; at the left a detached seta with its theca and diaphragm is clearly visible. Note the pigment in the shafts, their double lined contour, and the absence of tips from several setæ. The black spots in the verruca are the roots of ornamental hairs, some shafts of which are dimly seen at the right, among the setæ. $\times 100$.

FIG. 4. Schematic diagram of a seta. *Pigm.*, pigment plug; *Seta*, setal shaft; *P. d.*, poison duct; *Diaphr.*, diaphragm of the shaft; *Coll.*, collar of the seta; *P. s.*, poison sac, or theca; *Bulb*, cutaneous bulb containing the poison sac; *Bulb n.*, setigerous (?) nuclei; *V. r.*, vestigial root of the seta. (Cf. hair roots in other figures.) $\times 250$.

FIG. 5. Setal bulb and part of shaft, with diaphragm. Note the thickened collar, the hair root at the left of the bulb, the nuclei of the poison sac, and the small calcareous granules. Phosphotungstic acid-hematoxylin. $\times 450$.

PLATE 63.

FIG. 6. Urticating hairs of various European caterpillars, from Göldi, after Lampert. (a) "*Kupferglucke*," *Gastropacha quercifolia*; (b) nun-moth, *Ocneria monacha*; (c) another type of hair from *Gastropacha quercifolia*; (d) woolly-bear, *Arctia caca*; (e) the peacock, *Vanessa io*; (f) European tussock-moth, *Orgyia antiqua*.

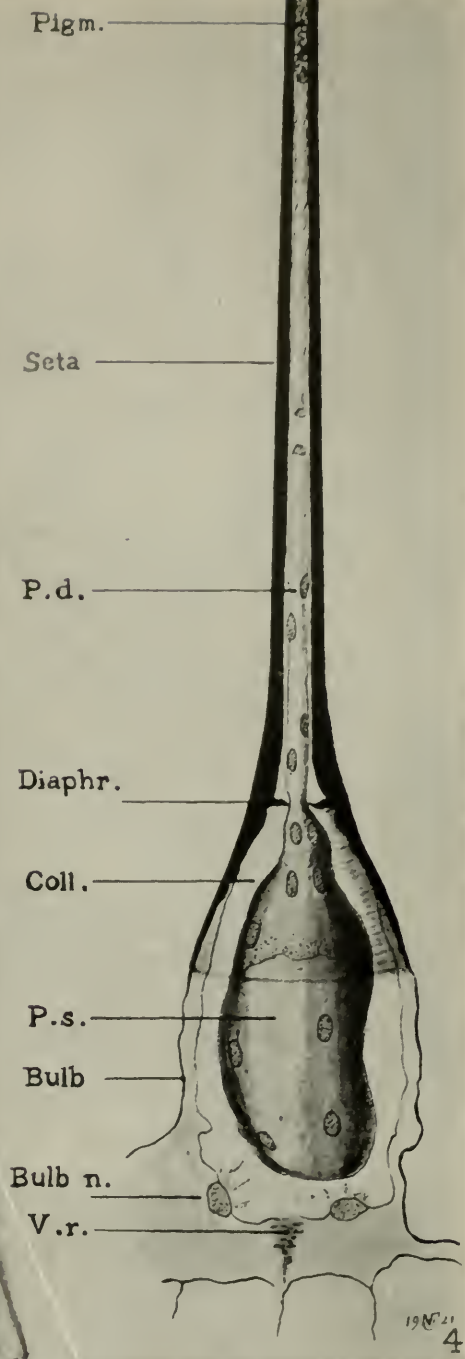
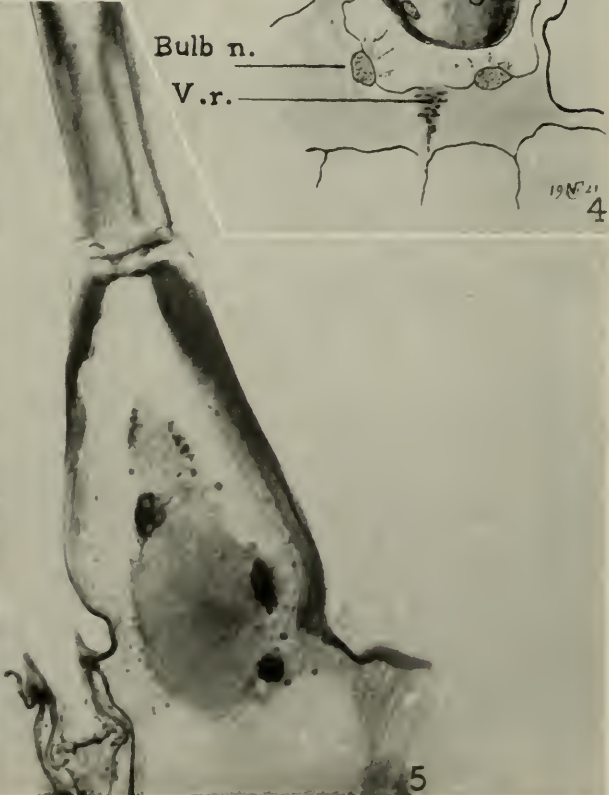
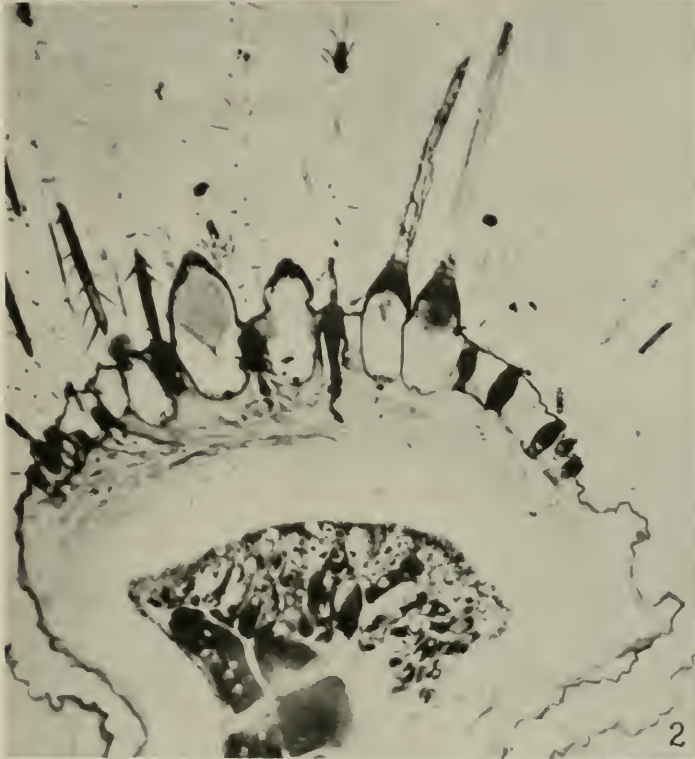
FIG. 7. Setal fragment in human epidermis. The coagulum about the fragment is chiefly cellular debris from the epithelium. The fragment is blunt at both ends and probably represents the tip of a seta that had been previously fractured at its point. Note the pycnosis of the neighboring nuclei and the beginning vesicle. Eosin-methylene blue. $\times 450$.

FIG. 8. Setal tip in a papular lesion, with a vesicle forming at its point and to the left. The compression of the epithelium in the immediate vicinity of the seta and the degenerated condition of its cells are quite marked. There are coagula at the point of entrance, which forms the apex of the papule, and at the tip of the seta. Phosphotungstic acid-hematoxylin. $\times 450$.

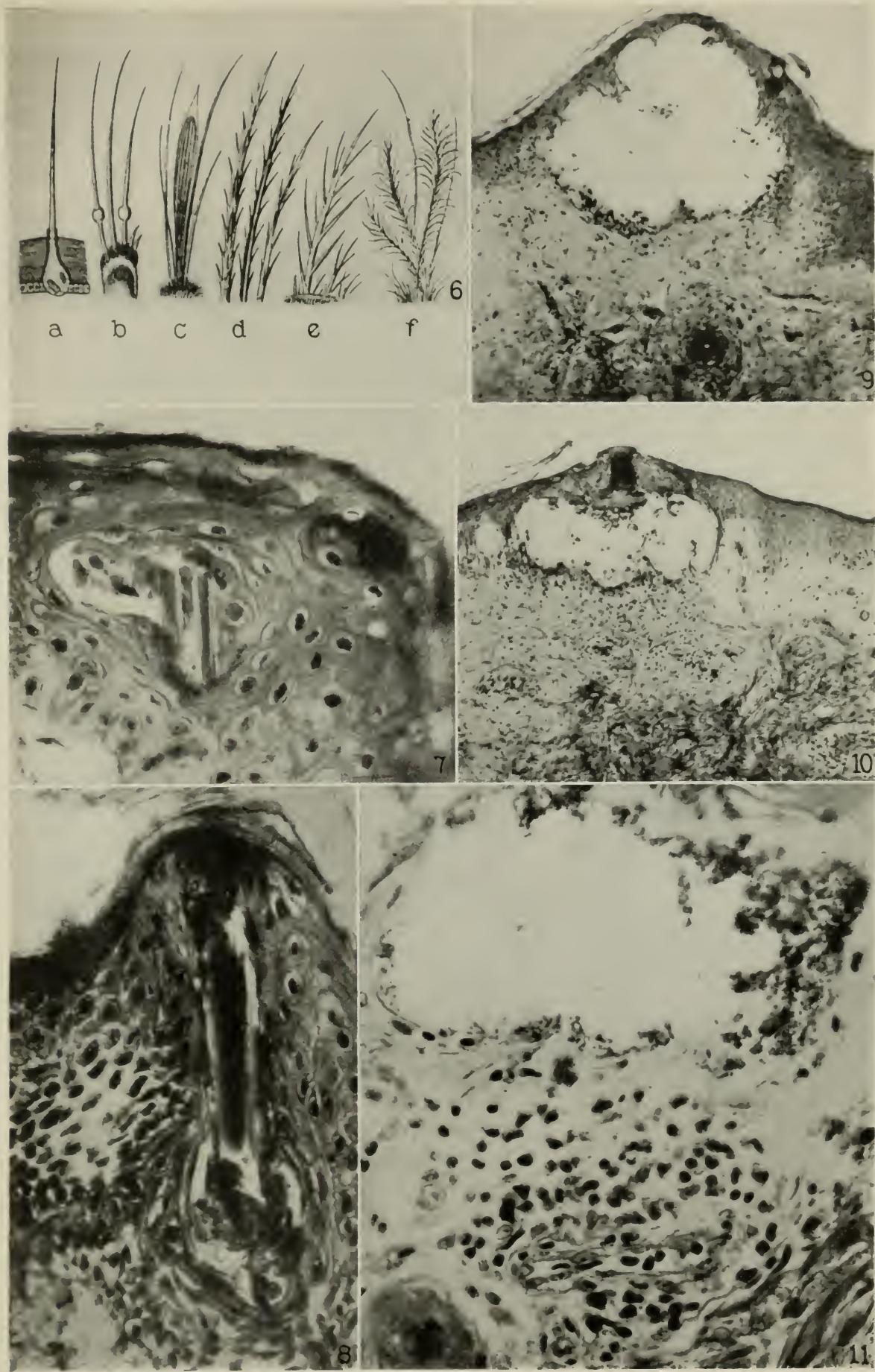
FIG. 9. Large vesicle to the left of a setal fragment, which is seen in the superficialities of the right wall, surmounted by a small, detached scab. Phosphotungstic acid-hematoxylin. $\times 100$.

FIG. 10. Section of another vesicle. The puncture wound referred to in the text is seen at the apex, plugged with coagulum. Strands of fibrin and lymphocytes are present in the vesicle, and the infiltration of the corium by the lymphocytes is well shown. Note the perivascular infiltration below, in the right hand corner. Phosphotungstic acid-hematoxylin. $\times 100$.

FIG. 11. Perivascular lymphocytic infiltration and distended lymphatic with fibrinous coagulum. Eosin-methylene blue. $\times 450$.



(Foot: *Megalopyge opercularis*.)



(Foot: *Megalopyge opercularis*.)

A TEN YEAR OLD STRAIN OF FIBROBLASTS.

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PLATES 64 TO 66.

(Received for publication, January 26, 1922.)

A strain of fibroblasts, obtained from the heart of a chick embryo on January 17, 1912, has completed the 10th year of its life *in vitro*. On January 17, 1922, our incubators contained about 60 cultures which represented the 1860th generation of the connective tissue cells. The purpose of this paper is to describe the actual condition of the strain, to discuss its meaning, and to summarize the findings.

1. *Present Condition of the Strain.*—The growth of the tissue fragments is as rapid today as during the past years. Each fragment generally doubles its volume in 48 hours. The cultures (Fig. 1) have not modified their appearance.¹⁻⁴ They are composed of typical fibroblasts which grow in a dense tissue (Fig. 2) at the periphery of which individual cells may be observed and photographed (Figs. 3 and 4). Many cells divide mitotically (Figs. 5 to 8).

2. *Unlimited Proliferation and Potential Immortality of Fibroblasts.*—The fact that fibroblasts have been kept in active condition for 10 years has an important significance. It demonstrates that tissue cells living *in vitro* transform the foodstuffs of their medium into protoplasm, and that their mass increases effectively. Some experimenters believed that cells cultivated outside of the organism did not use the substances contained in the medium,⁵ and that the mass of the tissue did not increase. This opinion applies to tissues cultivated in Locke

¹ Carrel, A., *J. Exp. Med.*, 1912, xv, 516.

² Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273.

³ Carrel, A., *J. Exp. Med.*, 1914, xx, 1.

⁴ Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.

⁵ Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277. Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421. Burrows, M. T., *Anat. Rec.*, 1916-17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

solution, or by the plasma method, but not to tissues cultivated in a medium containing embryonic tissue juices.⁶ Observation of our strain of fibroblasts shows this beyond all doubt. In 10 years, more than 30,000 cultures have been derived from a fragment of heart less than 1 c.mm. in size. If it had been feasible to multiply the tissues to their greatest possible extent, today their mass would be very much larger than the sun. Thus it is evident that some of the food material contained in the medium has been transformed into protoplasm.

The existence of the 10 year old strain demonstrates also that the cells are potentially immortal. This hypothesis was proposed long ago by Carrel when he observed that after 28 months of life *in vitro* the rate of growth of the active tissue cells had not decreased.³ It was evident that fibroblasts living under the conditions of his experiments were no longer subjected to the influence of time, as they are when living within the organism. They could be compared to unicellular organisms, such as *Paramecium*, which Woodruff has cultivated for over 14 years,⁷ and could be considered as potentially immortal. This opinion was criticized by Harrison,⁸ who considered it unjustified until the cells should have lived in cultures for a period exceeding the duration of life of the organism from which they were taken. Pearl⁹ believes that the present age of the strain of fibroblasts fully disposes of this criticism, as the limit of the life of chickens is often 10 years. It may be considered as certain, therefore, that fibroblasts will proliferate indefinitely, as do colonies of Infusoria.

3. *Use of the Strain of Fibroblasts in Physiological Studies.*—The purpose of the experiments was not only to ascertain whether fibroblasts could proliferate indefinitely, but also to study certain biological problems. Pure cultures of cells¹⁰ are as necessary in physiology as pure cultures of microorganisms in bacteriology. The strain, being composed only of typical fibroblasts, grows with great regularity, and was found to respond readily to changes in the composition of the culture medium by a modification of its rate of proliferation. The

⁶ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

⁷ Woodruff, L. L., cited by Pearl, R., *Scient. Monthly*, 1921, xii, 202.

⁸ Harrison, R. G., *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 71.

⁹ Pearl, R., *Scient. Monthly*, 1921, xii, 332, 333.

¹⁰ Carrel, A., *J. Exp. Med.*, 1912, xvi, 165.

original technique^{1,11} was progressively perfected. Studies of the optimum medium from the point of view of osmotic tension¹² and H ion concentration¹³ were made. Substitution of a fibrinogen suspension for plasma was rendered possible.¹⁴ More accurate methods of preparing and measuring the cultures were developed,¹⁵ by which differences of less than 10 per cent in the rate of growth could be ascertained.

The strain, then, could be used as a reagent for the detection of substances contained in the humors which have the power of activating or decreasing the rate of cell proliferation. The variations of the rate of growth demonstrated the presence in embryonic juice of a factor which increases the velocity of cell multiplication to a high degree.^{6,16} It showed also that the state of the fibroblasts at a given time was function of two independent variables, their dynamic condition at the preceding instant, and the condition of the medium.¹⁷ It became evident that the activity of the fibroblasts does not depend on the amount of potential energy they contained at the beginning of their life but upon certain substances present in the medium. The cells remained indefinitely young or grew old¹⁷ according to the food material they were given and the extent of the elimination of their catabolic substances.

In the same manner, it was found that adult serum produces on fibroblasts an effect opposite to that of embryonic tissue juice. This inhibiting power of a serum increased very much with the age of the animal from which it was obtained.¹⁸ As there was a definite relation between the age of the animal and the rate of cell proliferation in its serum, the growth index of the serum indicated the variations in the age.¹⁹

¹¹ Carrel, A., *J. Am. Med. Assn.*, 1911, lvii, 1611.

¹² Ebeling, A. H., *J. Exp. Med.*, 1914, xx, 130.

¹³ Fischer, A., *J. Exp. Med.*, 1921, xxxiv, 447.

¹⁴ Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

¹⁵ Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

¹⁶ Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

¹⁷ Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

¹⁸ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599; 1922, xxxv, 17.

¹⁹ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.

The strain was used also in the study of the response of tissue cells to an antigen. It is known that an antigen added to a culture of bone marrow determines the formation of an antibody.²⁰ When the strain was sensitized by a foreign protein, its response to a second injection²¹ showed that it could be used in the investigation of certain phenomena of immunity. As it has become possible lately to obtain strains of lymphocytes²² and of epithelial cells²³ living *in vitro* by practically the same procedure that is used for fibroblasts, the scope of these studies will be increased.

EXPLANATION OF PLATES.

PLATE 64.

FIG. 1. Culture 24871-1. Passage 1858. 10 years, lacking 4 days, of life *in vitro*. Portion of the central and peripheral zone. Among the peripheral cells, a number of mitoses can be distinguished. Stained with methylene blue after 48 hours growth. $\times 100$.

PLATE 65.

FIG. 2. Culture 24870-1. Passage 1858. 10 years, lacking 4 days, of life *in vitro*. Very active culture. Peripheral area in which nine mitotic figures can be observed. Stained with methylene blue after 48 hours growth. $\times 300$.

PLATE 66.

FIG. 3. Culture 24871-1. Passage 1858. Typical fibroblast in the peripheral zone of the culture shown in Fig. 1. $\times 1,000$.

FIG. 4.²⁴ Culture 422. Passage 1710. 9 years, 3 months, lacking 4 days, of life *in vitro*. Typical fibroblast. Stained with gold chloride after 48 hours growth. $\times 1,100$.

FIGS. 5 and 6. Culture 24041-2. Passage 1844. A cell undergoing mitotic division after 10 years, lacking 1 month, of life *in vitro*. Stained with methylene blue after 48 hours growth. $\times 1,000$.

²⁰ Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

²¹ Fischer, A., *J. Exp. Med.*, 1922, xxxv, 661.

²² Carrel, A., and Ebeling, A. H., unpublished experiments.

²³ Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

²⁴ I am indebted to Dr. Albert Fischer for permission to show a picture of this particular preparation.

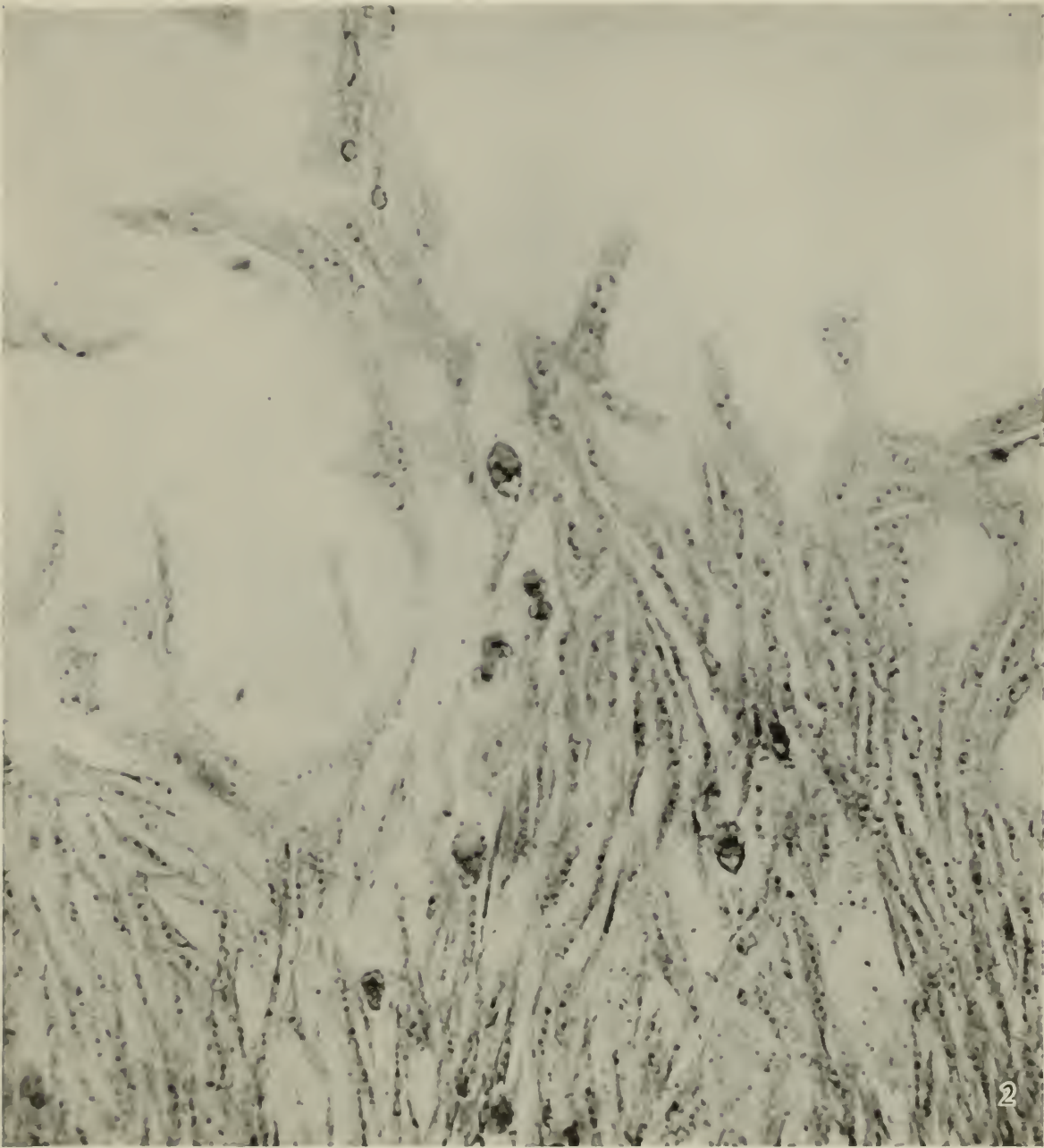
FIG. 7. Culture 964 A. Passage 1857. 10 years, lacking 2 days, of life *in vitro*. Fibroblast a short time after division. Stained with methylene blue after 48 hours growth. $\times 1,000$.

FIG. 8. Culture 964 A. Passage 1857. Double mitoses in an actively growing culture, after 10 years, lacking 2 days, of life *in vitro*. Stained with methylene blue after 48 hours growth. $\times 1,000$.

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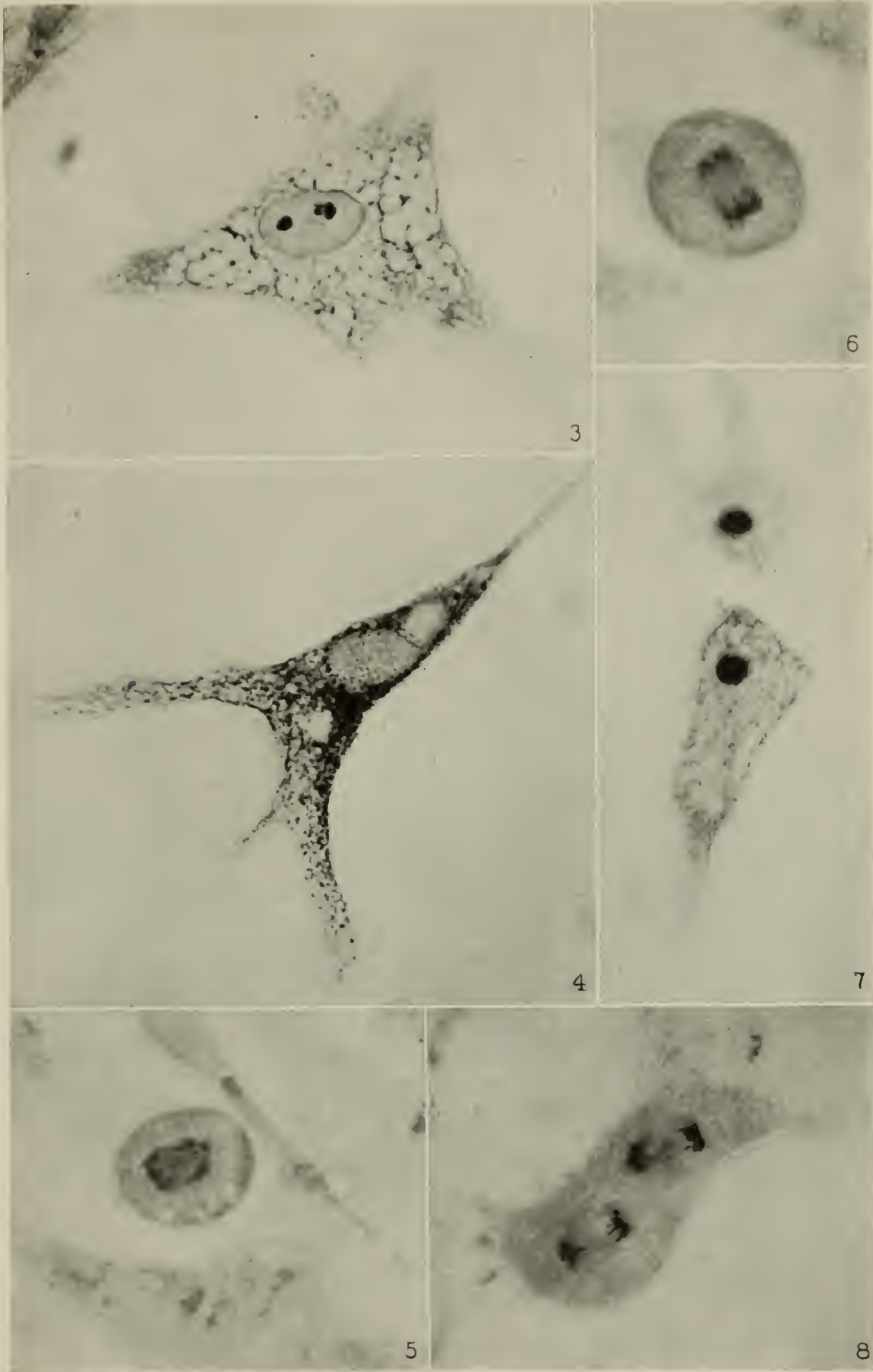


(Ebeling: Ten year old strain of fibroblasts.)



(Ebeling: Ten year old strain of fibroblasts.)

7608



(Ebeling; Ten year old strain of fibroblasts.)

STUDY OF THE ACTION OF FOUR AROMATIC CINCHONA DERIVATIVES ON PNEUMOCOCCUS. A COMPARISON WITH OPTOCHIN.

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(Received for publication, January 12, 1922.)

Since Morgenroth¹ introduced ethylhydrocupreine (optochin) as a chemotherapeutic agent against the pneumococcus, a number of related compounds has been investigated in the effort to find one more distinctly monotropic. However, up to the present, optochin holds a unique position in that it alone has been proven to influence beneficially an experimental pneumococcus infection in mice, in which animal the bacteriotropism is greater than the coincident organotropism. But this difference in the balance between organotropic and bacteriotropic action of optochin is insufficient to establish a definite therapeutic effect once the pneumococcus infection has become systemic in nature. The use of optochin against pneumococcus infection in man has had a rather comprehensive trial. In pneumonia, the results of investigations of Moore and Chesney,² Manliu,³ Wright,⁴ Parkinson,⁵ and others have shown its use in this connection to be of little value. This rather discouraging outcome has shown itself to be true, despite the increase in the bactericidal power of the patient's blood (Moore² and Wright⁴) and the negligible change in opsonic index (Wright⁴) following its use. Bier⁶ has claimed therapeutic effect in treatment of superficial wounds. Uncertain results in pneumococcus meningitis of man were reported by Wolff and Lehmann,⁷ Lippman,⁸ and Rosenow,⁹ while Cordua¹⁰ has found a favorable action in cases of

¹ Morgenroth, J., and Levy, R., *Berl. klin. Woch.*, 1911, xlviii, 1560, 1979. Morgenroth, J., *Naturwissenschaften*, 1913, i, 609. Morgenroth, J., and Bieling, R., *Berl. klin. Woch.*, 1917, liv, 723.

² Moore, H. F., and Chesney, A. M., *Arch. Int. Med.*, 1917, xix, 611; 1918, xxi, 659.

³ Manliu, J., *Berl. klin. Woch.*, 1916, liii, 58.

⁴ Wright, A. E., *Lancet*, 1912, ii, 1633, 1701.

⁵ Parkinson, C., *Z. Chemotherap., Orig.*, 1913, ii, 1.

⁶ Bier, A., *Berl. klin. Woch.*, 1917, liv, 717.

⁷ Wolff, S., and Lehmann, W., *Jahrb. Kinderheilk.*, 1914, lxxx, 188.

⁸ Lippman, *Berl. klin. Woch.*, 1917, liv, 781.

⁹ Rosenow, G., *Deutsch. med. Woch.*, 1920, xlvi, 9.

¹⁰ Cordua, R., *Berl. klin. Woch.*, 1921, xlviii, 1323.

meningococcus meningitis. However, Kolmer and Idzumi¹¹ were unable to produce a favorable influence on an experimental pneumococcus infection of the meninges of dogs and rabbits.

The realization of the limitations of this compound led us to investigate the cinchona derivatives synthesized and reported by Jacobs and Heidelberger.¹² It seemed to us that regardless of whether or not a substance was found that possessed greater activity than optochin, a biological study of these numerous compounds—so closely related to optochin—might be an aid in further chemical and biological investigations.

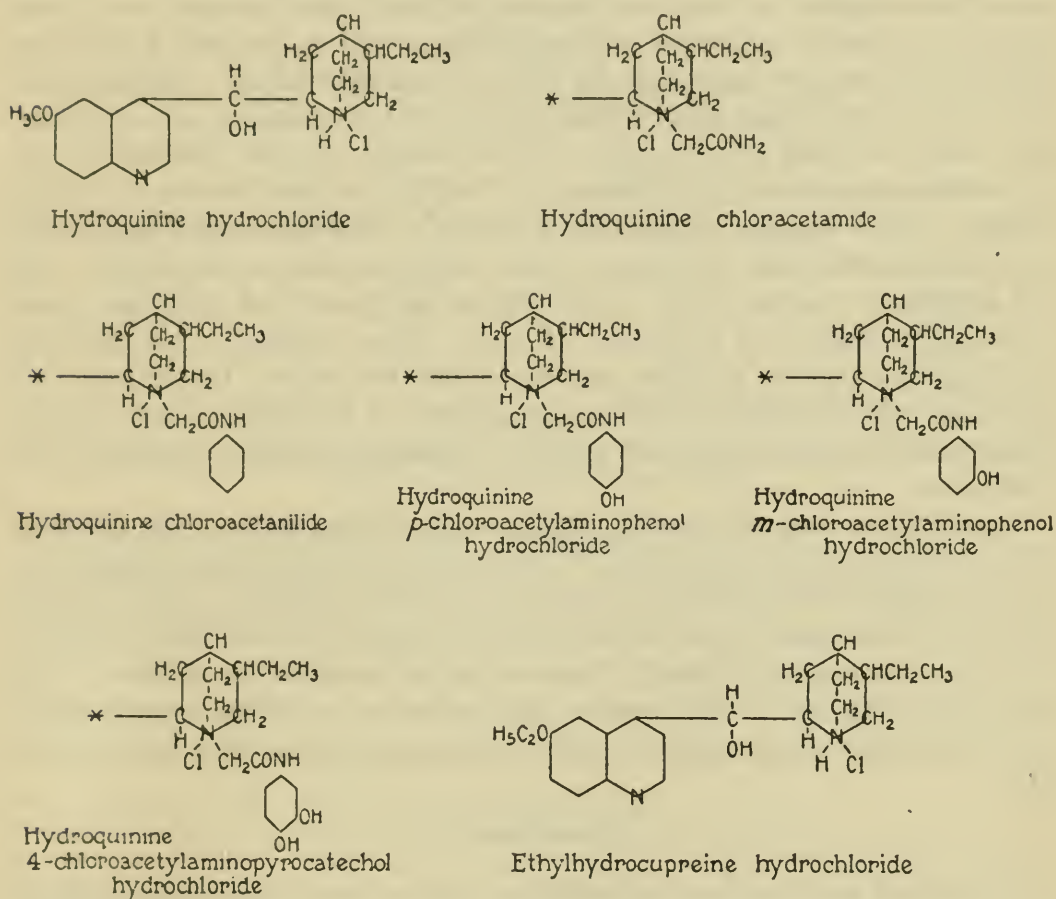
It was found at the outset that certain of these modified cinchona compounds possess a remarkably high bactericidal activity for pneumococci *in vivo*. In this paper we wish to present a study of four active chemicals in comparison with optochin. These four were chosen, as is shown below, for the reason that they represent a uniform series of hydroquinine derivatives, thus affording opportunity for a comparison between the chemical constitution and biological activity of closely related compounds.

To show clearly the relationship between these compounds and the exact chemical differences, structural formulas are given in Text-fig. 1. The first change in hydroquinine is the addition of chloracetamide on the quinuclidine ring, making hydroquinine chloracetamide. In turn, one of the amide hydrogens of the acetamide group is substituted by a benzene nucleus forming hydroquinine chloroacetanilide, the first important member of this series. The remaining drugs are hydroxy substitution products of the latter, in which the OH group is substituted in the para or the meta, or in both para and meta positions in the benzene nucleus, yielding respectively hydroquinine *p*-chloroacetylaminophenol hydrochloride, hydroquinine *m*-chloroacetylaminophenol hydrochloride, and hydroquinine 4-chloroacetylaminopyrocatechol hydrochloride. To avoid repetition of these names in this paper, the laboratory number will be used, as follows: the chloroacetylanilide, C 29; the *p*-chloroacetylaminophenol, C 36; the *m*-chloroacetylaminophenol, C 40; and the 4-chloroacetylaminopyro-

¹¹ Kolmer, J. A., and Idzumi, G., *J. Infect. Dis.*, 1920, xxvi, 355.

¹² Jacobs, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, 1919, xli, 2090.

catechol, C 110. As can be seen from the chart, these substances are derived from the hydroquinine and not from the ethylhydrocupreine nucleus, the only difference in the two compounds being that one is a methoxy and the other an ethoxy substitution on the quiniline nucleus. There is in these chemicals a combination of two bactericidal compounds—the quinines and benzenes. Since Lister introduced



TEXT-FIG. 1. Structural formulas of the cinchona alkaloids studied.

phenol into antiseptic surgery practically all the hydroxybenzene compounds have been employed for the same or similar purposes, but abandoned for one reason or another as impracticable. These new organic chemicals can therefore be thought of either as additive compounds of the benzene derivatives to the hydroquinine nucleus or the hydroquinine to the benzene nucleus by means of a common linkage CH_2ONH .

General Procedure.

Since we undertook to make a comparative study of the different chemicals, work was planned so that each experiment, in so far as was practical, embraced all the drugs under the same conditions. Young mice of a healthy stock, weighing from 15 to 18 gm., were employed throughout. Animals of this weight were used because it was early found that large, and especially old, female mice reacted irregularly. Doses of the chemical were approximated to 18 gm. mice. Each mouse was autopsied at death and one loop of heart's blood smeared on a blood agar plate, presence or absence of growth being noted at the end of 24 hours. Animals that survived were kept for 30 days and then killed. Cultures were made from a few animals living the 30 day period, but inasmuch as no pneumococci could be found, this procedure was abandoned. Unless otherwise stated in the tables pneumococci were present at death in the blood stream of all mice recorded. Since we found considerable variation in numbers of organisms in both the treated animals and the controls, little stress was laid on this factor. For each experiment, controls were run in duplicate with what had been found from previous experience to be 1, 10, and 20 M.L.D. This minimum lethal dose was calculated each time for the given experiment after the death of the controls, and was taken to be the smallest number of organisms, or the highest dilution of the culture (made in physiological salt solution), causing the death of duplicate mice in 48 hours.

The chemicals were dissolved in boiling sterile distilled water. The pneumococcus used was a Type I (Neufeld).¹³ Its virulence was such that generally two to four organisms (fluctuations occurring from time to time from two to twenty organisms) of a 6 hour culture, as determined by plating, injected into the peritoneal cavity of a mouse, resulted in the death of the animal within 48 hours. The intravenous virulence of this organism was rather low, requiring approximately 200,000 diplococci to cause death, or 10,000 times as many as by the intraperitoneal route.

Solubilities.

In Table I are given the solubilities of the chemicals in water, physiological salt solution, and serum. They have a practical bearing as can be seen below, in view of the bactericidal results obtained *in vivo* and *in vitro*. In making the determinations with water and physiological salt solution, each respectively was added slowly in measured amounts on 100 mg. of substance until solution was obtained. The solution in serum represents the concentration of the drug that does not cause precipitation in the serum, and can hardly be termed solubility. Both the aromatic compounds and optochin

¹³ The strain of pneumococcus was kindly furnished by Dr. O. T. Avery.

are protein precipitants. C 29 is the least and optochin the most soluble, while C 36, the para-hydroxybenzene derivative, is more soluble than C 40, with the OH group in the meta position. C 110, having the OH group both in the para and meta positions, holds an intermediate position between these two compounds.

TABLE I.
Solubilities.

Chemical.	Percentage of chemical in solvent.		
	Water.	Physiological salt solution.	Horse serum.
C 29	0.3	0.12	0.06
C 36	10.0	10.0	0.12
C 40	2.0	2.0	0.05
C 110	4.0	4.0	0.25
Optochin.	10.0	10.0	0.4

Bactericidal Action in Vitro.

Bactericidal action has been measured in two ways, (a) by a constant dose of organisms inoculated into varying concentrations of the chemical and (b) by a constant concentration of drug to which were added varying numbers of organisms. Since Chesney¹⁴ has shown

TABLE II.
*Bactericidal Action in Whole Blood in Vitro.**

Chemical	C 29	C 36	C 40	C 110	Optochin.
Dilution sterile in 2 hrs.....	1:1,500	1:750	1:1,000	1:500	1:8,000

* Neufeld pneumococcus.

that a young culture is more resistant to bactericidal agents than an old one, a 6 hour culture of the Neufeld pneumococcus was employed in these tests, in order that conditions might resemble as nearly as practicable those *in vivo*. In the first method whole defibrinated blood was used as medium.

¹⁴ Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

To 2 cc. of the blood was added the chemical in water solution with concentrations adjusted to give a constant volume in making the following series of dilutions: 1:500, 1:750, 1:1,000, 1:1,500, 1:2,000, 1:4,000, 1:8,000, and 1:12,000. This series was inoculated with 0.2 cc. of a broth culture per tube and incubated for 2 hours in a water bath at 37°C. One loopful from each tube was then distributed over one-fourth of a blood agar plate. These plates were incubated for 96 hours and read. From Table II it can be seen that under these conditions the aromatic derivatives are less active than optochin, the latter being about eight times as potent as C 29, the best of the others.

In the second method, bactericidal activity was determined by exposing the organisms in physiological salt solution, 50 per cent serum, and 50 per cent defibrinated rabbit blood, to the action of the chemicals for 5 minutes.

The different dilutions of pneumococci were made in 0.4 cc. of physiological salt solution, undiluted serum, and whole blood, respectively, by adding 0.1 cc. of the appropriate dilution of the culture to make the final concentration the one desired. The chemicals were dissolved in water, so that 0.5 cc. contained 1 mg. of substance. In the final test (Table III) there was a volume of 1 cc. with 1 mg. of chemical and a series of dilutions of a 6 hour culture of 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000. The controls in each case were made at the end of the experiment, 0.5 cc. of physiological salt solution being added to make the volume up to 1 cc. To each tube containing 0.5 cc. of culture was added 0.5 cc. of the chemical. The mixture was put in the water bath at 37°C. for 5 minutes, and then 0.05 cc. was pipetted into 100 cc. of meat infusion broth (pH 7.4), thus bringing the chemical dilution above its bactericidal potential (1:2,000,000). The flasks were under observation for 4 days and results recorded each 24 hour period except for the test on blood, the first observation in this case being made 12 hours after inoculation. Controls all grew out in 24 hours, while in this length of time no visible growth occurred in the flasks containing organisms subjected to the chemicals. The order of the rapidity of bactericidal action in decreasing ratio is as follows: C 29, C 40, C 36, optochin, and C 110. Neither serum nor blood diminished the activity of C 29 or optochin. The unchanged benzene nucleus derivative possesses the most rapid action; the one with the OH group in the meta position is more active than the one with the OH in the para position; while the compound with the OH group in both meta and para positions is still less active. Optochin in this experiment proves to have a slow rate of activity, as had been found by Solis-Cohen, Kolmer, and Heist.¹⁵

In addition, the dilution of the drugs that inhibited bacterial growth, with resultant death, was determined on meat infusion broth (pH 7.8).

¹⁵ Solis-Cohen, S., Kolmer, J. A., and Heist, G. D., *J. Infect. Dis.*, 1917, xx, 313.

TABLE III.
Bactericidal Action with a 5 Minute Contact Period. 1 Mg. of Chemical Used in Physiological Salt Solution,
Serum, and Whole Blood.

Chemical.	Dilution of pneumococcus (Neufeld).	Physiological salt solution.						50 per cent horse serum.						50 per cent defibrinated rabbit blood.					
		Chemical.			Control.			Chemical.			Control.			Chemical.			Control.		
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	24 hrs.	48 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	12 hrs.	24 hrs.
C 29	1:10	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:1,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:10,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
C 36	1:10	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:1,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:10,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
C 40	1:10	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:1,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:10,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
C 110	1:10	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:1,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:10,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
Optochin.	1:10	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:1,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:10,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+
	1:100,000	0	0	0	0	+	+	0	+	0	0	+	+	0	0	0	0	+	+

0.1 cc. of a 6 hour culture was pipetted into each tube (10 cc. volume) of broth containing the series of dilutions represented in Table IV. The inoculated broth was incubated for 4 days, and then 1 cc. from the last tube showing no visible growth was plated for confirmation. Hydroquinine (C 9) and hydroquinine chloracetamide (C 20) were added to the experiment for comparison.

It is rather interesting to note that the substitution of the chloracetamide on the hydroquinine nucleus decreases its bactericidal activity. With the exception of optochin, the same relationship persists between this group of chemicals as in the preceding experiment. C 29 is as active as optochin under these conditions.

TABLE IV.
Bactericidal Action in the Presence of Broth (pH 7.8).

Chemical.	Dilution in broth.														
	1:2,500	1:5,000	1:10,000	1:15,000	1:20,000	1:30,000	1:40,000	1:60,000	1:80,000	1:120,000	1:160,000	1:240,000	1:320,000	1:480,000	1:640,000
C 9							.	+	++	++	++	++	++	++	++
C 20		++	++	++	++	++	++	++	++	++	++	++	++	++	++
C 29											+	++	++	++	++
C 36								++	++	++	++	++	++	++	++
C 40										++	++	++	++	++	++
C 110				+	++	++	++	++	++	++	++	++	++	++	++
Optochin.											++	++	++	++	++

Toxicity.

Instead of the toxic dose the largest tolerant dose is given, as determined by averaging several titrations (Table V). The injections *per os* were made by means of a small silver stomach tube attached to a syringe by flexible rubber tubing, the volume injected always being 0.5 cc. From the standpoint of the largest tolerant dose, the same relative positions of the aromatic compounds exist organotropically as were shown to exist parasitotropically; that is, with these four aromatic compounds toxicity runs parallel with bactericidal power—the most toxic, C 29, is the most bactericidal. If rapidity of bactericidal action is used as the criterion, this is also true of optochin. The intravenous toxicity is perhaps largely a physical phenomenon due to protein precipitation.

In the balance between the organotropism and parasitotropism lies the hope of chemotherapy. It is perhaps unreasonable to suppose that a drug will ever be discovered that is strictly monotropic in its action. Manifestly, all the drugs used in clinical medicine show distinctly a therapeutic dose beyond which they set up complex phenomena causing aggravation rather than amelioration. Just as a drug is active often because of its affinity for a certain monocellular tissue in the animal body, so must we look for a drug that has a greater toxicity for monocellular organisms—bacteria—than for any cells vital to the defensive mechanism of the animal. It is perhaps impossible, at this stage of our knowledge, to make this comparison. However, if the bactericidal action of the largest tolerant dose of these

TABLE V.
Toxicity.

Chemical.	Largest tolerant dose.			
	Intravenous.	Intraperitoneal.	Subcutaneous.	<i>Per os.</i>
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
C 29	0.2	0.5	2.0	5.0
C 36	0.5	2.5	5.0	10.0
C 40	0.2	1.5	3.0	8.0
C 110	0.5	4.0	6.0	12.0
Optochin.	0.6	3.0	4.0	10.0

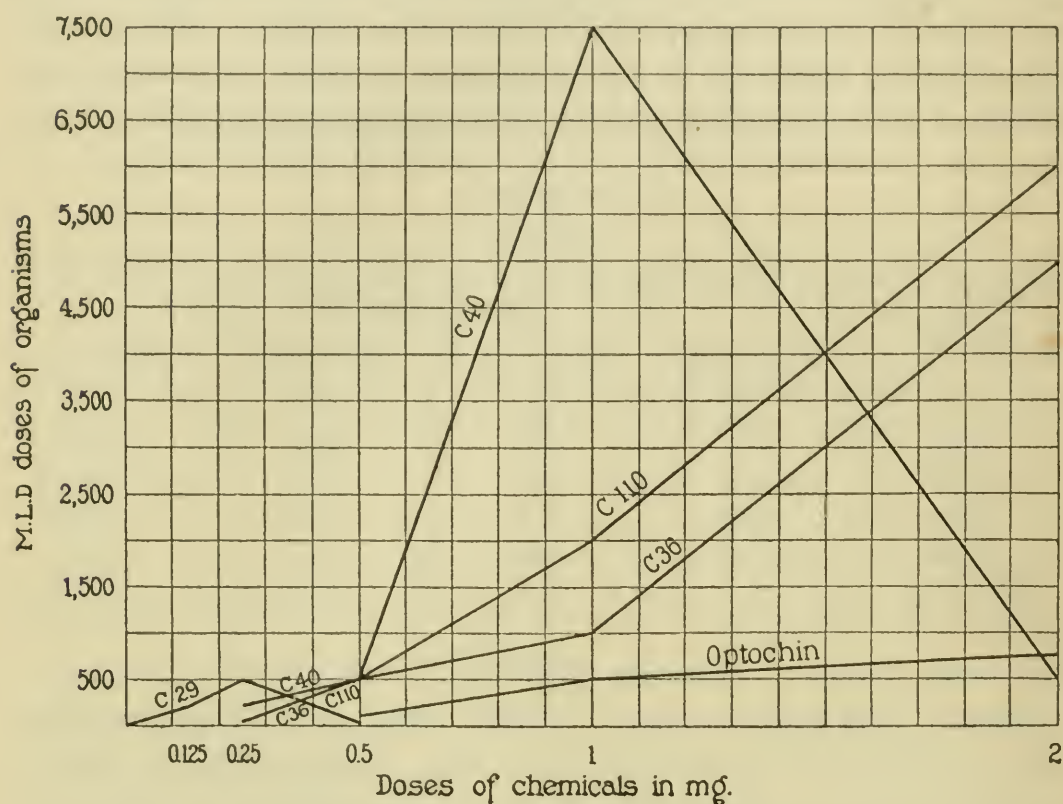
chemicals (Table V) for the experimental animal—the mouse—is compared with the number of virulent pneumococci that are killed *in vitro* (Table III) in as short a time as 5 minutes, the first step of this comparison is taken; that is, a non-toxic dose of the drug (for mice) is capable of killing *in vitro* many multiple lethal doses of virulent organisms. The following experiments were designed to show whether these relationships exist *in vivo*.

We wish to present the results of these *in vivo* experiments from a twofold standpoint. First, will the drugs kill multiple lethal doses of virulent pneumococci when chemical and organism are injected simultaneously into a walled off body cavity, such as the peritoneal? Second, will the drugs diffuse, and influence the experimental infection when injected by routes other than the one by which the animal was

infected? Briefly, is there either a local or a systemic bactericidal action *in vivo*, or both?

Simultaneous Injection of Multiple Lethal Doses of Pneumococci with Varying Amounts of Drugs.

The simplest possible procedure, perhaps, by which to estimate *in vivo* bactericidal activity resulting in an animal following the injection of a given drug is a simultaneous injection of organism and



TEXT-FIG. 2. Representation of the number of minimum lethal doses of organisms influenced by different doses of drugs—a therapeutic zone phenomenon.

chemical into the peritoneal cavity. By using as an indicator a microorganism of known virulence, it is possible to obtain within limits of biological accuracy an estimation of the number of organisms killed. The curves depicted in Text-fig. 2 are only approximate, owing to the wide range in dosage of organisms. These curves represent a titration of the number of minimum lethal doses of pneumococci killed by each of four doses of drugs, decreasing by halves from the

largest non-lethal dose. The number of minimum lethal doses of organisms, namely 1, 10, 100, and 1,000, in the preliminary titrations, was the same for all the doses of each chemical. Subsequently dilutions representing minimum lethal doses intermediate between the above doses were injected to furnish data given in Text-fig. 2. All titrations were made in duplicate.

The work with the aromatic compounds gave consistent results throughout. Optochin, on the other hand, varies in its effectiveness, especially in the 2 mg. dose. With this dose, the 740 M.L.D. from which the mice were protected is the average of five titrations: 400, 100, 200, and 1,000 to 2,000 M.L.D. The last two figures were obtained when the pneumococcus was at its greatest virulence, two to four organisms causing the death of the control in 48 hours. At the time of the other three experiments, 20 to 40 organisms were necessary to cause death of the controls in this interval. The fact that approximately ten times as many organisms were present in the unit injected at one time as at the other possibly accounts for the variable results. As the aromatic compounds are more rapid in their action, this may account for the fact that the results are more constant than with the more slowly acting optochin.

Hydroquinine has been shown by Morgenroth, Kolmer,¹⁵ and Tugendreich and Russo¹⁶ to have slight but distinct therapeutic value, though it is not so active as ethylhydrocupreine. By using the same technique as employed with these aromatic derivatives, it was found that hydroquinine protected against 100 M.L.D. at a time when this was represented by approximately 200 pneumococci. Hydroquinine chloracetamide, in the same experiment, protected against only 10 M.L.D. The results with these two chemicals show a parallelism between *in vitro* and *in vivo* bactericidal action (Table IV).

The very striking zonal reaction noted with C 29 and C 40 shows that there is an optimum concentration of the chemicals, below their toxic dose, at which virulent pneumococci are killed *in vivo*. If a therapeutic amount larger or smaller than this is injected, the mice die of septicemia. Manifestly, whatever the mode of action in this case may be, the normal defensive mechanism of the animal

¹⁶ Tugendreich, J., and Russo, C., *Z. Immunitätsforsch., Orig.*, 1913, xix, 156.

is weakened, the relation between mono- and polytropism being reversed. This same phenomenon occurs with the other chemicals, as will be shown later in this paper.

Comparison of Single and Repeated Injections of the Same Amount of Drug.

To make a still more vigorous test on this local effect, experiments were carried out on single and repeated injections of drugs, 1 and 2 hours after the animal was experimentally infected.

It has been shown by many workers that it is more difficult to cure an infection if time elapses between inoculation of the animal and application of remedial measures. That this is true with these chemicals is shown in Table VI. The experiment thus represented was carried out to show the relative effectiveness between single doses, and the same amount divided into fifths. These two methods of treatment were applied to mice 1 hour after injection of organisms, those receiving a single dose of drug being injected with 10 M.L.D., while those having the dose divided were injected with 10, 20, and 50 M.L.D.

The single dose of the drug proved ineffective, except with optochin, in which case one mouse with 10 M.L.D. survived. The repeated divided dose presented a different picture, as many as 50 M.L.D. of the organisms being killed with the aromatic compounds. Optochin in these small doses was unable to cure an animal infected with more than 10 M.L.D. The drop in the effectiveness of the chemicals when injected 1 hour after the organism is remarkable. The simultaneous procedure with C 40, for instance, proved to be lethal for 8,000 M.L.D. of organisms; injections deferred for 1 hour had no influence on 10 M.L.D. The power of the drug was thus decreased approximately 8,000 times.

Inasmuch as optochin, under these conditions, did not hold up as well as the other derivatives, the possibility was suggested that dosage was at fault. To establish this point and to determine the optimum concentration for five hourly injections, the experiment represented in Table VII was carried out.

2 hours after the injection of the pneumococci, a series of five injections of each doses of the chemicals, at intervals of 1 hour, was begun. The repeated 1 mg. doses of C 36 and C 40 were toxic for the mice. However, at death the C 36 animals had pneumococci in their blood streams, while with C 40, the animal with

TABLE VI.
Comparison of Single and Repeated Divided Doses of Drug, Given 1 Hour after Injection of the Organism.

Chemical.	Length of life.						
	After single dose of chemical.			After repeated doses of chemical, five times at hr. intervals.			
	Dose.*	No. of mice.	No. of M. L. D.*	Dose.*	No. of mice.	No. of M. L. D.*	
	mg.		10		10	20	50
C 29	0.5	4	24 hrs.; 9 days; 16 days; 24 days.	mg.	L.; L.†	144 hrs.; L.	L.; L.
C 36	1.0	4	60 " 72 hrs.; 36 hrs.; 120 hrs.	0.1	" "	L.; L.	" "
C 40	1.0	4	24 " 72 " 48 " 60 "	0.2	" "	" "	" "
C 110	2.0	4	72 " 240 " 96 " 168 "	0.2	" "	" "	" "
Optochin.	2.0	4	72 " 120 " 140 " L.	0.4	" "	48 hrs.; L.	48 hrs.; 48 hrs.
Controls.	0	6	0.00000001 cc., 48 hrs. = 1 M. L. D.	0.4	" "	0.00000001 cc., 48 hrs. = 1 M. L. D.	

* Both drug and organisms were injected intraperitoneally.

† In the tables L. indicates lived.

TABLE VII.
Intraperitoneal Varying Doses of Drug Repeated Hourly for 5 Hours, Beginning 2 Hours after Injection of the Organism.
Titration of Chemicals.

Chemical.	No. of M. L. D.	No. of mice.	Length of life after varying doses.			
			1.0 mg.	0.5 mg.	0.25 mg.	0.125 mg.
C 29	10	2		36 hrs.; 24 hrs.	36 hrs.; 36 hrs.	10 days; L.
	100	2		24 " 24 "	36 " 36 "	36 hrs.; 36 hrs.
C 36	10	2	24 hrs.; 24 hrs.	36 " L.	L.; L.	
	100	2	24 " 36 "	60 " "	36 hrs.; 96 hrs.	
C 40	10	2	24 " 24 "	36 " 60 hrs.	L.; L.	
	100	2	36 " 24 "	36 " 36 "	60 hrs.; L.	
C 110	10	2	L.; L.	L.; L.	L.; L.	
	100	2	36 hrs.; L.	60 hrs.; L.	36 hrs.; 36 hrs.	
Optochin.	10	2	L.; L.	7 days; L.	48 " 48 "	
	100	2	60 hrs.; L.	36 hrs.; 60 hrs.	36 " 36 "	
Controls.	0	4	Succumbed to 0.0000001 cc. = 1 M. L. D.			

TABLE VIII.
Intraperitoneal Doses of Drug Repeated Hourly for 5 Hours, Beginning 2 Hours after Injection of Varying Doses of the Organism.
Titration of Organisms.

Chemical.	Dose.	No. of mice.	Length of life.		
			No. of M. L. D.		
			10	20	40
	mg.				
C 29	1.25	4	L.; L.; L.; 96 hrs.	36 hrs.; 36 hrs.; 36 hrs.; 36 hrs.	36 hrs.; 36 hrs.; 36 hrs.; 36 hrs.
C 36	0.25	4	96 hrs.; 96 hrs.; 96 hrs.; 24 hrs.	L.; L.; L.; L.	36 " 36 " 36 " L.
C 40	0.25	4	L.; L.; L.; L.	96 hrs.; 36 hrs.; 36 hrs.; 36 hrs.	36 " 36 " 36 " 36 hrs.
C 110	0.5	4	36 hrs.; L.; L.; L.	36 " L.; L.; L.	36 " L.; 36 hrs.; L.
Optochin.	1.0	4	36 " " " "	L.; L.; L.; L.	36 " 36 hrs.; 96 hrs.; 96 hrs.

the 10 M.L.D. gave a sterile heart's blood culture, that of the 100 M.L.D. mouse being positive. All other mice of this experiment proved to have a septicemia at death. It can be seen with repeated, as with single injections, of C 29 and C 40, that there is a definite optimum concentration at which these drugs act; in addition, C 110, C 36, and optochin show this zonal phenomenon with repeated doses, C 110 and optochin having a broader zone than the other chemicals. Optochin, however, as in the preceding experiment, did not influence more than 10 M.L.D. when administered in small (0.25 mg.) repeated doses.

In the experiment represented in Table VIII, we estimated the number of minimum lethal doses of pneumococci killed by the optimum concentration of the drug. The results parallel those represented in Table VII, and in addition show that C 36 and C 110 and optochin influence as many as 20 M.L.D. The irregularity seen with C 36 has occurred in other experiments. Often with a small number of organisms, the infection proceeded as in the control mice, while a larger number of organisms was killed.

Diffusion of Drugs.

Thus far our work indicates that within certain definite limits these cinchona compounds have marked *in vivo* bactericidal activity. Drugs to be effective in an infectious disease should have more than this local action. They must be able to penetrate or diffuse throughout the animal body and perhaps in the last analysis maintain a definite concentration in the blood stream. This is especially true with the experimental infection of pneumococci in mice, which is a typical septicemia and in no way comparable to a lobar pneumonia. With this idea in view, the following experiments were carried out.

Subcutaneous Administration of Drugs Following Intraperitoneal and Intravenous Injection of Pneumococci.—This is the method which Morgenroth and Levy¹ and Moore¹⁷ used in their investigations with optochin on mice. Their results show that optochin does diffuse into the circulation, inasmuch as an experimental infection was controlled in its incipency.

Table IX is a record of three experiments in which the drug was administered subcutaneously, and the organism injected either intraperitoneally or intravenously. In the first experiment duplicate mice were given 1 and 10 M.L.D.

¹⁷ Moore, H. F., *J. Exp. Med.*, 1915, xxii, 269, 389, 551.

TABLE IX.
Subcutaneous Injection of Drug.

Chemical.	Organism injected intraperitoneally.			Organism injected intraperitoneally.			Organism injected intravenously.		
	Dose.	No. of M. L. D.	Length of life.	Dose.	No. of M. L. D.	Length of life.	Dose.	No. of M. L. D.	Length of life.
C 29	mg.			mg.			mg.		
	0.5	1	L.; L. 48 hrs.; 36 hrs.	2.0	1	48 hrs.; 48 hrs.	0.5	$\frac{1}{2}$	60 hrs.; 48 hrs.
		10			10	48 "		1	48 "
C 36	1.0	1	L.; L. 36 hrs.; 36 hrs.	4.0	1	36 " 48 "	1.0	$\frac{1}{2}$	48 " 96 "
		10			10	36 " 48 "		1	48 " 48 "
C 40	1.0	1	L.; L. 36 hrs.; 48 hrs.	2.0	1	L.; 48 hrs.	1.0	$\frac{1}{2}$	L.; L.
		10			10	48 hrs.; 48 hrs.		1	60 hrs.; 60 hrs.
C 110	2.0	1	36 " 48 "	4.0	1	48 " 48 "	2.0	$\frac{1}{2}$	60 " 48 "
		10	36 " 36 "		10	48 " 48 "		1	60 " 60 "
Optochin.	2.0	1	L.; L. 48 hrs.; L.	4.0	1	L.; L.	2.0	$\frac{1}{2}$	L.; 60 hrs.
		10			10	" 96 hrs.		1	60 hrs.; 48 hrs.

of pneumococci intraperitoneally, followed immediately by the drug. There seem to be indications that the drugs, save C 110, do have a systemic distribution in sufficient quantity to influence the progress of the attack of virulent organisms. This action is slight, however, optochin having greater effectiveness than the other compounds. In the second experiment a large non-toxic dose was given to duplicate mice with 1 and 10 M.L.D. The infection was not influenced, except in the case of optochin. The same zonal phenomenon persisted that occurred with C 29 and C 40 and in fact all the aromatic compounds (Text-fig. 1); that is, a large non-toxic dose of the drug did not favorably influence the course of the infection, the mice, if anything, being more sick than those given smaller doses. The third experiment represents an attempt to influence the course of an experimental septicemia. Again in duplicate, mice were given $\frac{1}{2}$ and 1 M.L.D. (1:1,000 and 1:5,000) of a 6 hour culture of Neufeld pneumococci intravenously, followed immediately by the drug subcutaneously. It is seen that not only did the mice injected with 1 M.L.D. of the drug die, but all save those with C 40 and optochin of the $\frac{1}{2}$ M.L.D. series. Seemingly with this dose, at least, the natural defensive mechanism is interfered with, so that the animal receiving one-fifth of the number of pneumococci (approximately 40,000 organisms) necessary to cause the death of a mouse, died in the same time as the controls.

Intravenous Injection of Drug and Organism; Intravenous Injection of Organism and Intraperitoneal Injection of Drug, and Vice Versa.—If the subcutaneous injection of the drug decreased the ability of the animal to cope with virulent organisms, one might expect that injection of the drug intravenously would also destroy a part of this defensive process.

That this is the case is shown in the first experiment in Table X. Although optochin was not so toxic as the aromatic compounds, a single dose of the drug did not influence the progress of the infection with 1 M.L.D. of organisms. In the next experiment with the intravenous organism and intraperitoneal drug, the outcome was practically the same as with the intravenous injection of the organism and subcutaneous injection of the drug. Again not only does the intraperitoneal injection of the chemical not affect favorably an experimental septicemia in mice, but causes the animal to succumb to a number of organisms below the minimum lethal dose.

Diffusion of Drugs through the Gastrointestinal Tract.—Inasmuch as there has been found to be a definite therapeutic dose for these chemicals when given by the intraperitoneal and intravenous routes, varying amounts were employed to test their diffusibility from the gastrointestinal tract.

TABLE X.
Simultaneous Injection of Drug and Organism.

Chemical.	Both drug and organism injected intravenously.			Organism injected intravenously, drug intraperitoneally.			Organism injected intraperitoneally, drug intravenously.		
	Dose.	No. of M.L.D.		Dose.	No. of M.L.D.		Dose.	No. of M.L.D.	
		$\frac{1}{2}$	1		$\frac{1}{2}$	1		$\frac{1}{2}$	1
	mg.			mg.			mg.		
C 29	0.1	72 hrs.; 144 hrs.	60 hrs.; 11 days.	0.5	36 hrs.; 36 hrs.	36 hrs.; 36 hrs.	0.1	L.; L.	36 hrs.; 36 hrs.
C 36	0.2	48 " 120 "	36 " 36 hrs.	1.0	120 " 48 "	36 " 36 "	0.1	" 36 hrs.	36 " 36 "
C 40	0.1	36 " L.	36 " 7 days.	1.0	48 " 60 "	36 " 48 "	0.1	" L.	36 " 36 "
C 110	0.3	L.; L.	60 " 72 hrs.	2.0	L.; L.	60 " 72 "	0.3	" 36 hrs.	36 " 36 "
Optochin.	0.3	" "	120 " 48 "	2.0	" "	120 " 48 "	0.3	" 36 "	36 " 36 "

A series of four doses was used—both single and repeated—each being one-half the amount of the preceding (Table XI). Mice were injected intraperitoneally with 2 to 4 M.L.D. of organisms immediately followed by the drug *per os*. With the single dose there was demonstrated a diffusibility sufficient to influence this small number of pneumococci. As with the intraperitoneal injection of the drug, there is in this case also a concentration of chemicals above which the organisms are not killed, and the animal dies of septicemia. Although C 29 and C 110 delayed death, there were no actual survivals. C 36, C 40, and optochin did prevent the development of an infection, optochin, as before, being active in a large dose. On the other hand, repeated doses of all the drugs exerted a destructive influence upon the organism.

From this experiment it would be very difficult to say which one of the chemicals was the best. To determine this fact, the optimum concentration of the drug was used in another experiment with multiple lethal doses of pneumococci (Table XII).

Three doses of the chemical were given at 2 hour intervals, beginning immediately after the injection of the organisms. Although there was very little variation in effect of the different chemicals, results prove that inasmuch as there were some survivals, some action other than purely local was exerted. In the same table are presented the results concerning the effectiveness of drugs after intravenous injection of the organism. It happens that by this method of administration the same destructive action of the defensive mechanism of the animal does not occur with C 40, C 110, and optochin, as occurred by intraperitoneal route. In fact, with these compounds, there was a survival of the mice injected with 1 M.L.D. This experiment with intravenous injection of the organism was repeated, with multiple minimum lethal dose, but no more than 1 M.L.D. was influenced with C 40, C 110, and optochin, while with C 29 and C 36, death of the mice ensued on less than 1 M.L.D.

The rapidity with which the bactericidal activity of these compounds is neutralized within the animal body is shown in Table XIII.

A therapeutic dose of each drug was injected into the peritoneal cavity of mice, in triplicate, and 5 minutes later, 1, 10, and 20 M.L.D. of pneumococci were injected by the same route. An amount of chemical which in simultaneous injections kills 500 to 8,000 M.L.D. is fixed, changed, exhausted, or somehow destroyed in 5 minutes, so that all the mice injected with 10 M.L.D. died, and none receiving 20 M.L.D. survived.

TABLE XI.
Per Os Therapy after Intraperitoneal Injection of 2 M.L.D. of the Organism.

Chemical.	Single doses immediately after injection of organism.			Three doses, 2 hrs. apart; first immediately after injection of organism.		
	Dose.	No. of mice.	Length of life.	Dose.	No. of mice.	Length of life.
	mg.			mg.		
C 29	2.5	4	48 hrs.; 72 hrs.; 72 hrs.; 120 hrs.	2.5	3	24 hrs.; 24 hrs.; 48 hrs.
	1.25	3	48 " 48 " 96 "	1.25	3	24 " 24 " L.
	0.625	3	72 " 48 " 48 "	0.625	3	48 " 72 " "
				0.25	3	48 " L.; L.
C 36	5.0	4	48 hrs.; L.; L.; L.	5.0	3	24 " 24 hrs.; 36 hrs.
	2.5	3	72 " " "	2.5	3	24 " 48 " L.
	1.25	3	72 " " "	1.25	3	48 " L.; L.
				0.625	3	54 " " "
C 40	5.0	4	48 hrs.; 48 hrs.; 48 hrs.; 54 hrs.	5.0	3	24 " 48 hrs. 48 hrs.
	2.5	3	L.; L.; L.	2.5	3	24 " 48 " 48 "
	1.25	3	72 hrs.; L.; L.	1.25	3	48 " 48 " L.
				0.625	3	L.; L.; L.
C 110	5.0	4	48 hrs. 60 hrs.; 72 hrs.; 72 hrs.	5.0	3	24 hrs.; 24 hrs.; 48 hrs.
	2.5	3	48 " 96 " 72 "	2.5	3	24 " 48 " 60 "
	1.25	3	48 " 72 " 72 "	1.25	3	48 " L.; L.
				0.625	3	72 " " "
Optochin.	5.0	4	72 hrs.; L.; L.; L.	5.0	3	48 hrs.; 48 hrs.; 48 hrs.
	2.5	3	72 " 72 hrs.; 72 hrs.	2.5	3	48 " 72 " L.
	1.25	3	48 " 72 " 72 "	1.25	3	48 " L.; L.
				0.625	3	48 " 48 hrs.; L.

TABLE XII.

Per Os Therapy. Three Doses Repeated at 2 Hour Intervals; First Dose Simultaneous with Injection of the Organism.

Chemical.	Dose.		No. of mice.		Length of life.		No. of m. l. d. injected intraperitoneally.		
	mg.	No. of mice.	No. of m. l. d. injected intravenously.		Length of life.	No. of mice.	2	20	40
			k	1					
C 29	0.25	4	24 hrs.; 24 hrs.; 48 " 6 days.	24 hrs.; 48 hrs.; 48 " 72 "		4	48 hrs.; 48 hrs.; L.; L.	96 hrs.; L.; L.; L.	48 hrs.; 72 hrs.; 72 " 72 " 48 " 72 " 72 " L.
C 36	0.5	4	36 " 36 hrs.; 4 days; 6 days.	36 " 48 " 96 " 6 days.		4	48 hrs.; 72 hrs.; L.; L.	48 hrs.; 48 hrs.; L.; L.	
C 40	0.5	4	L.; L.; L.; L.	36 " 48 hrs. L.; L.		4	L.; L.; L.; L.	72 hrs.; L.; L.; L.	48 " 72 hrs.; 6 days; L.
C 110	1.0	4	" " "	L.; L.; L.; L.		4	48 hrs.; L.; L.; L.	48 hrs.; L.; L.; L.	48 hrs.; 48 hrs.; L.; L.
Optochin.	1.0	4	" " "	" " "		4	48 hrs.; L.; L.; L.	48 hrs.; L.; L.; L.	48 hrs.; 48 hrs.; 48 " L.
Controls.	0	4	0.0005 cc. 6 days; L. L.; L.	0.0001 cc. 36 hrs.; 48 hrs.; 96 hrs.; 120 hrs.		4	48 hrs.; 48 hrs.; 48 " 48 "	36 hrs.; 36 hrs.; 36 " 36 "	36 hrs.; 36 hrs.; 36 " 36 "

TABLE XIII.
Protection Experiment. Drug Injected 5 Minutes before the Organism.

Chemical.	Dose.	No. of mice.	Length of life.		
			No. of M.L.D.		
			1	10	20
C 29	mg. 0.5	3	36 hrs.; 36 hrs.; 36 hrs.	36 hrs.; 36 hrs.; 36 hrs.	36 hrs.; 36 hrs.; 36 hrs.
C 36	1.0	3	12 " 72 " 36 "	36 " 36 " 36 "	60 " 36 " 36 "
C 40	1.0	3	36 " L.; L.	36 " 48 " 240 "	36 " 36 " 36 "
C 110	2.0	3	36 " " "	36 " 72 " 120 "	36 " 72 " 72 "
Optochin.	2.0	3	48 " " "	36 " 72 " 72 "	36 " 60 " 72 "
Controls.	0	3	36 " 36 hrs.; 36 hrs.	36 " 36 " 36 "	36 " 36 " 36 "

Our work as so far reported has been restricted to a Type I pneumococcus (Neufeld). Moore² showed that there is a difference in the bactericidal action of optochin with pneumococci of the different types, but it is not necessarily specific for types. Using the same procedure of simultaneous intraperitoneal injection of organism and drug, described above in connection with the Neufeld pneumococcus, we have titrated an additional Type I and two each of Types II and III (Table XIV). It would appear from these results that the chemicals are most active against Type I, less so against Type II, and least against Type III. That this specificity perhaps is not as

TABLE XIV.

Comparison of the Relative Protection Afforded by Different Drugs against Multiple Lethal Doses of Type I, II, and III Pneumococci. Simultaneous Injection Intraperitoneally of Drug and Organism.

Chemical.	No. of M. L. D.					
	Type I.		Type II.		Type III.	
	Neufeld.	No. 15.	No. 28.	No. 32.	No. 14.	No. 27.
C 29	500	100	400	20	0	1
C 36	5,000	1,000	400	50	0	0
C 40	8,000	1,000	500	100	0	1
C 110	6,000	1,000	400	50	10	1
Optochin.	700	400	400	50	10	1
No of organisms per M.L.D.	4	18	9	8	700,000	5,000,000

marked as it would seem is borne out, even in the case of these six strains, by two considerations. First, the virulence of the Type III strains was not as great as that of either of the Type I or of the Type II, the Type III having a minimum lethal dose in one strain of 700,000 and in the other of 5,000,000 organisms. It is possible that we are dealing not with type specificity but with organisms more or less resistant to chemicals and of varying numerical degrees of virulence. This is emphasized in the experiment shown in Table XV. Then, during another investigation, the virulence of the Type I (Neufeld) was reduced, so that 1 M.L.D. contained 7,000 instead of two to four organisms. This organism was used in multiple lethal doses, 1, 10, 100, and

TABLE XV.
Effect of Drugs on a Neufeld Type I Pneumococcus of Lowered Virulence (Decreased Approximately 4,000 Times).

Chemical.	Dose.	No. of mice.	Length of life.			
			No. of m.l.d.			
			1,000	100	10	1
			No. of organisms.			
			6,500,000	650,000	65,000	7,000
C 29	mg. 0.5	2	48 hrs.; 48 hrs.	48 hrs.; 48 hrs.	96 hrs.; 112 hrs.	L.; L.
C 36	2.0	2	48 " 48 "	48 " 48 "	48 " 96 "	" "
C 40	1.0	2	48 " 48 "	48 " 48 "	L.; L.	" "
C 110	2.0	2	48 " 48 "	48 " 48 "	48 hrs.; L.	" "
Optochin.	2.0	2	48 " 48 "	48 " 48 "	48 " 48 hrs.	48 hrs.; 96 hrs.
Controls.	0	2	48 " 48 "	48 " 48 "	48 " 48 "	48 " 48 "

1,000, and followed immediately by the chemical (simultaneous method). The results with organism in this stage of activity were very similar to those with Type III above, the chemicals, except C 40, being active against not more than 1 M.L.D.

Rabbit Experiment.

Two experiments were carried out with rabbits (Table XVI). Organisms and drugs were injected simultaneously. Regardless of toxicity, 25 mg. per kilo of each chemical were used. The pneumococcus (Neufeld) in a preliminary titration demonstrated a virulence such that 0.000001 cc. of a 6 hour serum broth culture

TABLE XVI.
Rabbit Experiment.

Chemical.	Pneumococci 0.00001 cc.					Pneumococci 0.000001 cc.				
	Dose per kilo.	Rabbit No.	Weight.	Life.	Culture.	Dose per kilo.	Rabbit No.	Weight.	Life.	Culture.
	mg.		gm.	days		mg.		gm.	days	
C 29	25	1	2,300	3	++	25	9	2,100	4	++
C 36	25	2	1,900	11	++	25	10	1,775	L.	
C 40	25	3	1,540	L.		25	11	2,000	"	
C 110	25	4	1,850	7	++	25	12	2,020	"	
Optochin.	25	5	1,950	2	++	25	13	1,675	3	++
	25	6	2,000	3	++	25	14	2,200	3	++
Controls.	0	7	1,800	3	++	0	15	1,950	3	++
	0	8	2,100	3	++	0	16	2,100	3	++

killed a 2 kilo rabbit in 3 days. In the first experiment with 0.0001 cc., or 100 M.L.D., the rabbit treated with C 40 remained alive (30 days), those treated with C 36 and C 110 showed a slight delay, while the controls, the C 29, and the optochin animals succumbed in 2 or 3 days. In the second experiment the same procedure was carried out, except that 1 cc. of 0.00001 dilution of organisms was employed for injection. The C 29, optochin, and control animals died in 4, 3, and 3 days respectively, those of C 36, C 40, and C 110 survived.

These results show that the bactericidal activity of the aromatic compounds is not restricted to mice. Optochin seemingly does not act with sufficient rapidity in rabbits to kill this number of organisms *in vivo*. The results of this experiment confirm the work with optochin of Moore,¹⁷ Scott,¹⁸ and Lewis.¹⁹

¹⁸ Scott, W. M., *J. Path. and Bact.*, 1914-15, xix, 130.

¹⁹ Lewis, J. H., *Arch. Int. Med.*, 1918, xxii, 593.

DISCUSSION.

The aromatic compounds described are a series of chemicals possessing rapid bactericidal action both *in vitro* and *in vivo*. There is, however, a pronounced variation in their action from the bacteriotropic and organotropic standpoint. C 29 ranks first in rapidity of action *in vitro*, but because of its polytropic characteristics is the poorest *in vivo*. The para-hydroxy substitution product (C 36) is an improvement in that the decrease in bactericidal action is more than compensated for by the lessening of its toxicity for mice. The bactericidal activity *in vitro* of the meta-hydroxy compound (C 40) is almost as great as the unsubstituted benzene derivative (C 29), and its toxicity is reduced 50 per cent. The dihydroxybenzene compound (C 110), judged by its ability to kill multiple lethal doses of pneumococci *in vivo*, holds an intermediate position between the para and meta derivatives. Its *in vitro* bacteriotropic activity, however, is much less than that of both the other aromatic chemicals and optochin. This substance is an example of a drug which, when injected simultaneously with multiple lethal doses of a virulent pneumococcus into the peritoneal cavity of mice, causes the death of a greater number of organisms than a corresponding amount *in vitro*. Apparently in a therapeutic dose its activity is auxiliary or perhaps stimulatory to the other natural forces of an animal which combat an infection. From the above it may be seen that there is a relationship between chemical constitution and chemotherapeutic activity.

The ultimate test of a chemotherapeutic agent is whether its use results in the cure of an established infection. Mice are unsatisfactory animals with which to make this test. The incubation period for the pneumococcus is so short, as well as the length of life following a fatal dose of the organism, that any remedial measure, if treatment be delayed till the infection is systemic in nature, may give misleading results.

Our method of investigation has shown that when pneumococci and drugs are injected simultaneously into the peritoneal cavity the death of multiple lethal doses of the organisms results. Any delay in the administration of the drugs neutralizes their effectiveness. Although an experimental infection was arrested when treatment was

postponed 2 hours after the injection of multiple lethal doses of organism (with chemicals and pneumococci injected intraperitoneally), at no time have we been able to cure a mouse having a systemic infection. The treatment involving the longest delay was administered after an 8 hour interval. In this experiment, not reported above, mice were injected with 1 M.L.D. and treatment was begun 8 hours later. 60 per cent of the mice treated with the aromatic compounds, with the exception of C 29, survived. Inasmuch, however, as pneumococci did not appear in the blood stream of the controls until 12 hours had elapsed, this study merely proves the chemicals to have a local therapeutic action, though of a high degree of efficiency. However, that there is some therapeutic activity resulting from diffusion of the drugs, as tested by injecting chemicals and organisms through different routes, is shown by the favorable results obtained in *per os* therapy.

Binz,²⁰ in an extensive series of experiments, has shown that, along with other physiological and pharmacological attributes of certain of the cinchona alkaloids, (1) they are general protoplasmic poisons, (2) stimulating in small amounts the activity of ameba, Infusoria, and white blood cells, in larger amounts paralyzing, and (3) decreasing the oxidizing and reducing power of the blood and weakening the enzymotic action. The summary of his work indicates that quinine in small amounts stimulates normal functional activity, in large amounts causing paralysis of this same mechanism. We are unable to say that these aromatic derivatives produce the same effect, but it has been shown that in experiments with pneumococcus infection of mice their action is zonal; that is, within certain dosage the bacteriotropic action of the chemicals is greater than the deleterious influence on the defensive mechanism of the animal. Amounts above this therapeutic dose, although non-lethal, paralyze processes vital for the animal's defense, permitting the invading organism to develop without restraint. It is difficult to understand why this large, non-lethal dose does not kill the bacteria, when seemingly more than a sufficient quantity to do so is injected. There is an apparent reversal of relationship from bacteriotropism with small doses to organotropism with the large. In other words, there is a greater affinity for the animal

²⁰ Binz, C., *Virchows Arch. path. Anat.* 1869, xlv, 67; *Das Chinin. Nach den neuern pharmakologischen Arbeiten dargestellt*, Berlin, 1875.

tissue than for the pneumococci, when large amounts of chemical are injected. This zonal phenomenon was found to occur in intraperitoneal, subcutaneous, and *per os* administration of all the drugs, optochin having a less pronounced zonal effect than the aromatic compounds.

The question arises as to whether it would be advisable to use drugs for chemotherapeutic purpose that present this zonal phenomenon, which at first glance seems so discouraging. That this may not be an insuperable barrier is suggested by the fact that it is present in some degree in all drugs used in clinical medicine. Drugs in general have a therapeutic zone beyond which treatment cannot be carried with safety to the patient. The practical value of any therapeutic agent lies in its ability to produce desired effects in relatively non-toxic quantities.

The question naturally arises whether the aromatic cinchona compounds which are reported in this paper may have therapeutic applications in man. This cannot be answered until further and wider studies have been made. Our experiments have been confined almost entirely to the one animal species; but that other larger animals can be used may be inferred from the experiments successfully carried out on rabbits. That the list of synthesized pharmacological agents effective against a bacterial infection has been enlarged is obvious. But we feel that the time for the employment of any of the drugs here described in man has not yet arrived. Further investigations along the lines followed may not impossibly lead to the goal being sought.

CONCLUSION.

It has been shown with one strain of pneumococcus (Type I, Neufeld), that hydroquinine chloroacetanilide (C 29), hydroquinine *p*-chloroacetylaminophenol hydrochloride (C 36), hydroquinine *m*-chloroacetylaminophenol hydrochloride (C 40), and hydroquinine 4-chloroacetylaminopyrocatechol hydrochloride (C 110) have a rapid pneumococcal activity both *in vitro* and in the peritoneal cavity of mice, and to a lesser extent in rabbits. In comparison, optochin is slower in action, but its power is not so easily destroyed either *in vitro* or *in vivo*.

The introduction of the hydroxy group of the benzene nucleus of hydroquinine chloroacetanilide changes the relationship between

organotropism and bacteriotropism. In comparing the rapidity of *in vitro* bactericidal action and intraperitoneal toxicity, C 29 exhibits the most rapid pneumococcal action and is the most toxic for mice. C 36, the para-hydroxy derivative, is one-fifth as toxic as C 29 and only one-tenth less active bactericidally. C 40 is one-half as toxic and has approximately the same bactericidal power, while C 110 is one-eighth as toxic and one-fifth as pneumococcal; and optochin is one-sixth as toxic and has one-fifth the bactericidal action. Arranged in the order of their ability to kill pneumococci when injected simultaneously with them into the peritoneal cavity, the drugs are: C 40, C 110, C 36, optochin, and C 29.

The chemotherapeutic action of the aromatic compounds is essentially local in character. But by *per os* therapy there is demonstrated a certain amount of diffusion of this activity, not shown by any other method of administration, C 40 and C 110 having about the same value as optochin.

Intravenous injection of the drugs in small doses destroyed to a greater or less extent the natural defenses of the animal, optochin being perhaps less injurious than the aromatic compounds. This same destruction of natural resistance followed intraperitoneal and subcutaneous injections of the chemicals as measured by intravenous injections of the organisms.

The maximum tolerant dose in a single injection (intraperitoneal) is not so efficacious as the same dose divided in fifths and injected at hour intervals. Optochin under these conditions is not so active as the aromatic compounds. In general, repeated doses are more curative than single.

There is a zone between the therapeutic and toxic doses, both single and repeated, for all these chemicals alike, where the natural resistance of the animal to an infection is reduced. This effect is noted especially with C 29, C 36, and C 40. In the case of optochin the therapeutic dose is nearer the toxic than with C 110, C 36, and C 40. Apparently these chemicals exhibit a variability in *in vivo* bactericidal activity according to different strains of pneumococci and numerical virulence.

We wish to thank Dr. W. A. Jacobs and Dr. M. Heidelberger for the supply of chemicals and other assistance.

A FURTHER INQUIRY INTO THE SOURCE OF THE VIRUS
IN BLACKHEAD OF TURKEYS, TOGETHER WITH
OBSERVATIONS ON THE ADMINISTRATION OF IPECAC AND OF SULFUR.

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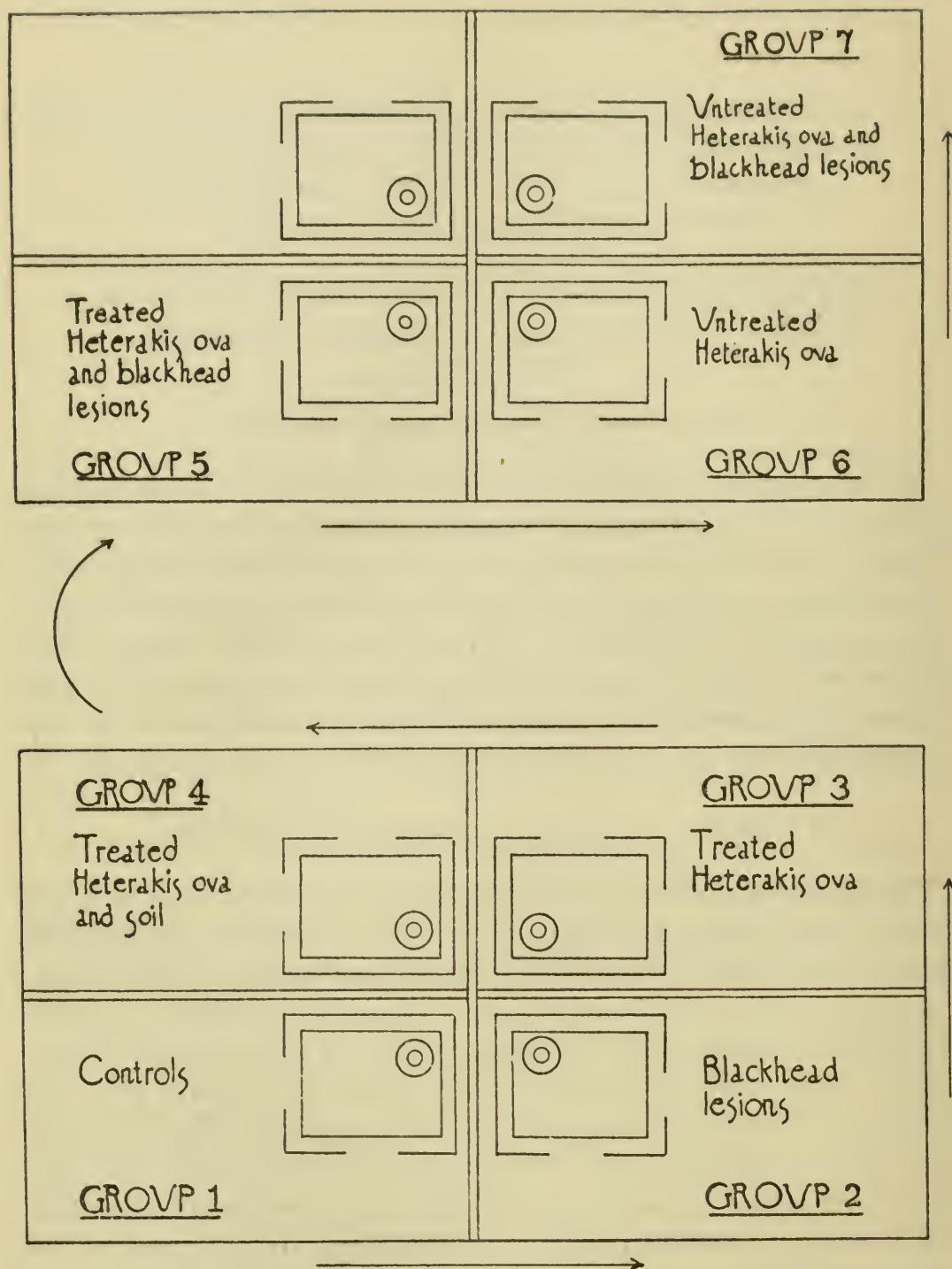
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The investigation of blackhead in turkeys has been continued during the past season with two main objects in view: first, to obtain further evidence concerning the source of the virus in the natural transmission of the disease; and second, to test the efficiency of certain drugs in the prevention or treatment of this infection. Other observations with respect to contraction of the disease from the soil, especially that of hen yards, the occurrence of the disease in chickens, and the immunity following recovery from infection will also be discussed.

The Source of the Virus in Natural Transmission.

The following experiment was planned to determine if possible the source of the protozoan parasite of blackhead, *Histomonas meleagridis*, and more especially whether, in the natural transmission of blackhead, it is derived from the intestinal worm, *Heterakis papillosa*. A series of thirty-one turkeys from a single incubation was used in the experiment. These were divided into seven groups on removal from the incubator, each group being provided with a separate clean compartment in a laboratory room. They all received sterilized food and water, and were otherwise protected from any protozoan contamination throughout the experiment. The general arrangements of the compartments in which the turkeys were kept is shown in Text-fig. 1, and, as the value of the experiment lay in attention to minute details to remove any question of outside contamination, the outfit used will be described rather fully.



TEXT-FIG. 1. Plan of the compartments showing the distribution and treatment of the seven groups of turkeys used in Experiment 1. The arrows indicate the sequence in which the groups were fed and attended.

The compartments, which were also used in later experiments, were constructed by building intersecting partitions of wallboard on the tops of two large tables and an enclosing wall consisting of a double strip of cheese-cloth, fastened to posts at the four corners of each table top and to the ends of the partitions. In order to eliminate flies and other insects, a mosquito bar was stretched over the top and tucked inside the cheese-cloth strip. The legs of the tables were placed in dishes of kerosene oil to isolate the compartments from any crawling insects. A simple hover was provided in each compartment with a 15 watt electric light enclosed in a tin can to furnish heat. The hovers consisted of rectangular frames with legs at the four corners, across which were stretched wires and over these were hung strips of heavy cotton flannel. The top was covered with the same material. In order to retain the heat, a pasteboard box was placed over each hover and openings were made on two sides for the turkeys to go in and out. It was necessary to see that the turkeys were all safely under the hovers at the end of the 1st day after removal from the incubator but thereafter no further attention in this respect was required.

Before each experiment, the mosquito bar, cheese-cloth, hovers, and cans were autoclaved, the partitions given a fresh coat of paint, and the table tops covered with many thicknesses of fresh clean paper. New boxes were provided for the hovers. Drinking fountains and food dishes were either autoclaved or sterilized by boiling. All materials entering into the diet were sterilized. Each compartment was furnished with a litter of sterile hayseed. Hard boiled eggs were fed throughout the greater part of the experiment. Sterilized grit was kept constantly on hand and after a few days pasteurized milk, soured by inoculation with a stock culture of *B. bulgaricus*, and sterilized chick feed were added to the diet.

The attendant was required to wash her hands thoroughly with soap and water and then in 70 per cent alcohol before feeding the turkeys. The food was dropped or poured into each compartment and, in case anything was touched, the hands were rinsed in alcohol before proceeding to the next compartment. Any dish removed from the compartment was sterilized before it was returned.

The above precautions were taken in order to eliminate any possibility of the introduction of *Histomonas* except in the additional materials which were intentionally furnished to the various groups.

Experiment 1.—Thirty-one turkeys hatched on June 2, 1921, were removed from the incubator the following day, divided into seven lots or groups, each lot being placed in a separate compartment and furnished with sterilized food and water as described above.

When 4 days old, the turkeys of six groups were given their first dose of the various materials, the infectiousness of which was to be tested. Group 1 received nothing additional and served as controls; Group 2 was fed liver lesions from acute blackhead cases; Group 3 received ripe *Heterakis* ova that had been treated for 3 days with 1.5 per cent by volume of nitric acid; Group 4 received similarly treated *Heterakis* ova and was also given a sod of clean lawn grass; Group 5 received both the treated *Heterakis* ova and liver lesions; Group 6 was

fed untreated *Heterakis* ova only; and Group 7 received untreated *Heterakis* ova and liver lesions. These various materials were fed on a number of occasions, the dates of which are given in Table I. The groups were at all times fed in numerical order beginning with the controls (Text-fig. 1). This sequence in feeding was adopted intentionally to lessen the chance of contamination from any possible carelessness in carrying out directions. The attendant thus first fed the normal birds, then those which might possibly develop blackhead, and finally those which according to previous results would be most likely to develop the disease.

As each turkey died or was killed, a thorough postmortem examination was made, and the cecal contents were carefully examined for both worms and protozoa. The data of the experiment are furnished in detail in Table I.

That the measures taken to prevent accidental contamination of the stock employed in this experiment were adequate is shown by the postmortem findings in the group of control turkeys which was kept under the conditions already outlined for periods longer on the average than those in any of the other groups. None of the control turkeys showed any lesion, intestinal worm, or any form of protozoon.

The second group, which had been fed liver lesions fresh from cases of acute blackhead on three separate occasions, furnished two cases (50 per cent) of blackhead. One of these turkeys (No. 20), killed 15 days after the first feeding, showed typical blackhead; the other (No. 18), killed 21 days after the first feeding, showed a distribution of lesions that had hitherto never been encountered. As both ceca appeared to be normal throughout, presumably a large lesion of the proventriculus represented the primary focus, and smaller lesions in the liver metastases. Contact lesions resulting from the extension of the disease from liver lesions to the contiguous serous surface of the proventriculus are not infrequent, but in this case there was extensive necrosis of the mucosa without any visible involvement of the serous coat. A coccidium (*Eimeria avium*) was also found in three of the four turkeys of this group. Whether this very resistant organism was derived from some of the turkey eggs from which the birds were hatched, or from the diseased livers which were fed to them, or from air-borne dust, could not be determined. It should be mentioned that, of the various compartments, the one containing these birds was nearest a window (8 feet) which was kept open more or less all the time for ventilation. While this is suggestive of air-

borne infection the other possibilities are quite as plausible. The important point, however, is that the feeding of considerable amounts of blackhead virus may of itself produce infection in the absence of *Heterakis papillosa* or other parasitic worms.

The development of blackhead in all the turkeys of the third group furnishes very suggestive evidence as to the origin of the virus in the natural transmission of the disease. The previous treatment of the *Heterakis* material, *i.e.* subjection for 3 days to the action of 1.5 per cent nitric acid, rendered this material sterile with respect to bacteria (as shown by cultures) so that the survival of the protozoon, *Histomonas meleagridis*, outside of the *Heterakis* ova in this material is extremely improbable. This material, however, did produce typical blackhead in fatal form in each of the five turkeys to which it was fed, and the symptoms of the disease appeared as early as in the cases in which untreated *Heterakis* material was employed.

In case the virus was external to the membrane which so adequately protects the *Heterakis* embryos in the ripe eggs of this worm, it would be reasonable to expect at least a dilution effect as the result of the treatment with acid. Even if it were conceivable that the albuminous matter present did furnish a certain amount of protection, the frequent agitation in a large amount of acid and the subsequent washings in sterile salt solution would tend to eliminate any virus not included in the ova. All previous experiments indicate that the organism of blackhead is of a frail, non-resistant character. Inoculation is successful only when freshly obtained lesions are used, although at a low temperature (5°C.) the latter may remain active at least 4 days. Although the organisms may be maintained in an active state for a few days in appropriate culture media, they soon disappear completely, leaving no forms to be interpreted as resistant stages.

The possibility that the parasite of blackhead is transmitted through the turkey's egg to the young turkey is not wholly eliminated. However, if such were the case one would at least expect the disease to appear occasionally in isolated control turkeys, especially in those not rare instances in which pathological conditions as indicated by fibrinous cores are present in the ceca of otherwise healthy birds.

The demonstration that the feeding of the acid-treated *Heterakis* ova is all that is necessary for the production of blackhead leaves the

TABLE I.

Group.	Turkey No.	Treatment.	Death.†	Anatomical findings.	<i>Heterakis</i> .‡	Protozoa.
1 Controls.	11	None, other than isolation and a diet of sterilized food.	1921 June 21. K.	Normal.	0	None.
	12		" 30. "	"	0	"
	13		" 30. "	"	0	"
	14		" 25. "	"	0	"
	15		" 30. "	"	0	"
	16		" 27. "	"	0	"
2 Fed blackhead lesions.	17	June 6, 8, and 18, 1921. Fed fresh liver lesions.	June 27. K.	Normal.	0	<i>Eimeria avium</i> .
	18		" 27. "	Atypical blackhead, primary in proventriculus, secondary in liver.	0	<i>Histomonas</i> and <i>Eimeria avium</i> .
	19		" 28. "	Normal.	0	<i>Eimeria avium</i> .
	20		" 21. "	Typical blackhead.	0	<i>Histomonas</i> .
3 Fed treated <i>Heterakis</i> ova.	21	June 6 and 9, 1921. Fed <i>Heterakis</i> ova treated for 3 days with 1.5 per cent nitric acid.	June 24. D.	Typical blackhead.	21	<i>Histomonas</i> .
	22		" 23. K.	"	36	"
	23		" 25. D.	"	7	"
	24		" 25. K.	"	3	"
	25		" 28. D.	"	9	"
4 Fed treated <i>Heterakis</i> ova; soil.	26	June 6 and 9, 1921. Fed <i>Heterakis</i> ova treated for 3 days with 1.5 per cent nitric acid. Given sod from lawn.	June 24. D.	Typical blackhead.	7	<i>Histomonas</i> .
	27		" 27. "	"	9	"
	28		" 28. K.	"	2	" (pulsating forms).
	29		" 22. "	"	20	"

5 Fed treated <i>Heterakis</i> ova and blackhead lesions.	30	June 6 and 9, 1921. Fed <i>Heterakis</i> ova treated for 3 days with 1.5 per cent nitric acid. June 6, 8, and 18. Fed fresh liver lesions.	June 27. K. " 28. D. " 27. " " 7. K.	Typical blackhead. " " " " Cecal but no liver lesions.	12+§ 70+§ 17 6	<i>Histomonas</i> . " " "
	31					
	32					
	33					
6 Fed untreated <i>Heter-</i> <i>akis</i> ova.	34	June 6 and 9, 1921. Fed untreated <i>Hete-</i> <i>rakis</i> ova.	June 25. D. " 25. " " 28. " " 24. K.	Typical blackhead. " " " " " "	28 19 5† 24	<i>Histomonas</i> . " " "
	35					
	36					
	37					
7 Fed untreated <i>Heter-</i> <i>akis</i> ova and black- head lesions.	38	June 6 and 9, 1921. Fed untreated <i>Heter-</i> <i>akis</i> ova. June 6, 8, and 18. Fed fresh liver lesions.	June 24. K. " 28. " " 28. D. " 28. K.	Typical blackhead. " " " " " "	132+§ 5 11† 15	<i>Histomonas</i> . " (few pulsating forms). <i>Histomonas</i> . " (numerous pulsating forms).
	39					
	40					
	41					

* Turkeys hatched June 2, 1921.

† K. indicates killed; D., died.

‡ In the *Heterakis* column + indicates worms present; ++ worms in moderate numbers; +++ worms numerous; in combination with a number + indicates worms present in excess of that number.

§ Microscopic larvae also noted.

additional factors introduced in the four remaining groups without significance. Thus it is unimportant whether soil, virus derived from blackhead lesions, or fecal material is present with *Heterakis* ova, for the result in all cases is the same. The sixteen turkeys of these various groups all showed typical blackhead.

The groups fed acid-treated ova showed somewhat fewer *Heterakis*, visible to the naked eye (average 17), as compared with the groups fed untreated ova (average 30), but the individual distribution within the groups varied greatly, one turkey of a given group showing only 5, another 132 worms.

The finding of *Heterakis* in various stages of development, and especially the occurrence of small larval forms, weeks after the *Heterakis* material was fed, indicates that many of the ova pass through the alimentary tract of the young turkey intact and that these, on being again picked up from the litter in which food becomes scattered, may then hatch and develop.

In the ceca of several of the turkeys which survived longest in this experiment, rounded protozoa having the general appearance of small sized *Histomonas* were observed to pulsate rhythmically though weakly. This observation was suggestive of the occurrence, late in the infection, of a flagellated stage of *Histomonas*, but the point could not be definitely determined at this time because pulsating forms were found only in those turkeys which had been fed untreated *Heterakis* material or had been furnished soil.

The young turkeys were undoubtedly infected long before they showed any pronounced symptoms. Possibly their confinement in a small space in which they were not subjected to the tiring effects of a more variable environment, such as they experience when allowed to roam free, was the cause of their surviving much longer than would be expected in view of their age.

Experiment 2.—A lot of eleven turkeys hatched on September 6, 1921, was kept in a clean compartment and furnished sterilized food and water. One found dead at the age of 5 days showed firm slender cores in the ceca without any thickening of the walls; two died at the age of 8 days, one being crop-bound, and the other showing a firm yellow core distending one cecum. In none of these was there any indication of blackhead infection. On September 15, two were moved to another clean compartment and during the next 24 hours were fed five doses of

ripe *Heterakis* ova (collected in August) that had been kept for a week in 1.5 per cent nitric acid. The six other turkeys of this lot were fed a portion of the same *Heterakis* material which had not been treated (two on September 15 and four on September 22).

Both turkeys receiving the *Heterakis* ova kept for a week in dilute nitric acid developed characteristic blackhead. One was killed 21 days and the other died 28 days after receiving the last dose of ova. They both showed few *Heterakis* of sufficient size to be seen with the unaided eye but scrapings of the cecal mucosa showed numerous young larvæ.¹

The turkey which died 28 days after being fed acid-treated *Heterakis* material was autopsied at once and showed in the ceca numerous motile flagellates, apparently representing a flagellated stage of *Histomonas*. No other protozoa were found in fresh preparation, stained film, or section. In scrapings of the cecal mucosa planted in ovomucoid, a medium in which *Tetratrichomonas gallinarum* is readily grown, the above organisms died out completely within 48 hours. In stained films the organisms were readily identified as *Histomonas meleagridis* and in a considerable proportion one or two short flagella were demonstrable.

With the view of obtaining early stages of blackhead for histological studies, two of the turkeys that received untreated *Heterakis* material were killed 8 days later, one 9 days, two 10 days, and one 11 days after the ingestion of ova. In one a single *Heterakis* larva was found, one showed four, and the others numerous young worms. Blackhead was found in only one of the turkeys, killed 10 days after ingestion of ova.

The results of this experiment confirm those of Experiment 1 with respect to production of blackhead with *Heterakis* ova treated with dilute (1.5 per cent) nitric acid. They also show that *Histomonas meleagridis* may occur in flagellated form late in the disease. Small *Heterakis* larvæ were again found long after feeding the ripe ova of this worm.

That only one of the six turkeys that were fed untreated ova and killed from 8 to 11 days later should show blackhead infection indicates that invasion of the tissues by the organism of blackhead does not always immediately follow the ingestion of the worm eggs. From the results of previous experiments it appears probable that others of this lot would have become infected if they had lived 1 or 2 weeks longer.

¹ Some of these larvæ were no larger or more differentiated than the stages obtained 5 days after feeding *Heterakis* ova by Dr. C. Uribe of this laboratory who is making a study of the life cycle of this worm

The Effect of Drugs on Blackhead in Turkeys.

Ipecac Treatment.—In the course of a previous investigation² emetine, the active principle of ipecac commonly employed in amebic dysentery, was administered at regular intervals in toxic doses to two turkeys inoculated with blackhead. This drug failed to prevent the development of the subcutaneous lesions in either case. While one of the turkeys eventually recovered it was not thought that this should be attributed to the emetine, since spontaneous recovery from inoculated blackhead had already been noted. In a series of nine turkeys infected with blackhead through inoculation during the present investigations, three have recovered without treatment.

The recent report of Wegeforth³ on the value of ipecac in the prevention and treatment of blackhead in turkeys has aroused widespread interest. It appeared quite possible that ipecac, as it contains active principles other than emetine, might be of value even though the latter had failed. Furthermore, a drug which failed to destroy organisms injected into the tissues might prevent natural invasion from the cecal contents especially if administered by mouth. It was to test the value of ipecac that the following experiments were planned.

Experiment 3.—Ripe *Heterakis* ova were fed on July 20, 1921, to fifteen of a lot of forty turkeys hatched on July 14. Wine of ipecac was then administered to four of these in doses of 5 drops daily to see if infection could be prevented.

The failure of these ipecac-treated turkeys to contract blackhead is of doubtful significance, for only two of the eleven control turkeys showed blackhead infection. The absence of worms in four of the latter and the small number found in others indicated that the dosage of *Heterakis* ova was too small to furnish uniform results. 23 days after the feeding of *Heterakis* ova one of the four ipecac-treated turkeys was killed and showed no evidence of disease or intestinal worms. The other three were used in the following experiment.

Experiment 4.—Twenty-three turkeys hatched on July 14, 1921, were used, three having previously received small daily doses of wine of ipecac as already explained. On August 11 the turkeys, now 28 days old, were placed out of doors in two adjacent cages, twelve in one and eleven in the other. As there was no *Heterakis* material on hand, both groups of turkeys were fed a dry mash to which dirt from a hen yard had been added in the proportion of eight parts of mash to

² Tyzzer, E. E., Fabyan, M., and Foot, N. C., *J. Infect. Dis.*, 1921, xxix, 268.

³ Wegeforth, H. M., and Wegeforth, P., *J. Pharmacol. and Exp. Therap.*, 1921, xvii, 249.

one of dirt. A shovelful of the same dirt was thrown into the cages from time to time.

The two groups of turkeys were treated similarly in every respect except that throughout the experiment ipecac was added to the food of the eleven confined in one of the cages, the twelve in the other cages serving as controls. In order that the effect of ipecac on inoculated blackhead might also be tested, one of the controls and one of the ipecac-treated turkeys had been inoculated on August 10. Powdered ipecac was employed and for the first 6 days was given in a dry mash, so that the turkeys, averaging at first 300 gm. in weight, would each get about 0.08 gm. per day. Since the mash was not all eaten and became somewhat scattered, the amount of ipecac actually taken was probably considerably less than the dose furnished.

Beginning on August 17 ipecac was added to the daily ration of sour milk in an amount equivalent to 0.1 gm. for each turkey. The average body weight was now 400 gm. Although the drugged milk was taken slowly it was all cleaned up each day so that the dosage of ipecac was now between 0.1 and 0.18 gm. On August 19 ipecac was added to the milk in an amount equivalent to 0.2 gm. per turkey and was no longer furnished in the mash. On August 29 the dose was doubled, but as the turkeys then refused the milk, it was necessary on the following day to return to the previous dose of 0.2 gm. Although the birds had by this time greatly increased in weight, several were already showing symptoms of blackhead.

The detailed results of these various procedures are given in Table II.

The results obtained from the two inoculated turkeys show that ipecac has no specific action on the organisms of blackhead, as it occurs in the tissues of the turkey, the course of the disease being the same in both control and treated birds.

It is evident from this experiment that ipecac administered as a prophylactic in large doses has a distinct effect in that treated turkeys remain healthy longer than untreated birds when subjected to conditions such as might be furnished by the ordinary farmyard. Thus 63 per cent of the controls had died before any death had occurred among the treated turkeys, and 70 per cent of the latter were alive at the death of the last of the control turkeys.

Since this retarding effect of ipecac on the incidence of the natural type of infection occurred, notwithstanding the absence of any effect on the inoculated disease, it seemed important to ascertain whether the ipecac treatment might not tend to affect the development of *Heterakis*. These worms were found, however, to be as numerous in the treated as in the control turkeys. The inoculated turkey

TABLE II.

Group.	Turkey No.*	Treatment.	Death.	Anatomical findings.	<i>Heterakis</i> .
			1921		
1 Controls.	42†	From Aug. 11, 1921, dirt from hen yard in food and in cage.	Aug. 27. D.	Inoculated black-head together with typical lesions of cecum and liver	300 (estimated)
	43		Sept. 1. "	Typical blackhead.	+
	44		" 8. "	" "	+
	45		" 3. "	" "	165
	46		Aug. 23. "	" "	81
	47		" 28. "	" "	+
	48		" 28. "	" "	+
	49		Sept. 1. "	" "	+
	50		Aug. 31. "	" "	+
	51		Sept. 3. "	" "	+++
	52		" 4. "	" "	+++
	53		Aug. 28. "	" "	++
2 Ipecac-treated.	54†‡	From Aug. 11, 1921, dirt from hen yard in food and cage.	Aug. 28. D.	Inoculated black-head; ceca normal.	356
	55		Sept. 23. "	Typical blackhead.	27
		From Aug. 11, 1921, powdered ipecac at first in doses of 0.08 gm., gradually increased to 0.2 gm.			
	56		" 3. "	" "	293+
	57		Alive.	(Symptoms of black-head; recovery.)	
	58		Sept. 12. D.	Typical blackhead.	+
	59		Oct. 6. "	" "	92
	60		Sept. 11. "	" "	+
	61		" 4. "	" "	+
	62		" 26. "	" "	150+
	63‡		" 2. "	" "	59
	64‡		" 10. "	" "	+

* Turkeys hatched July 14, 1921.

† Aug. 10. Breast inoculated with liver lesions of Turkey C. P. S. 21.65.

‡ Previously treated with wine of ipecac.

(No. 54) which received ipecac treatment, and in which the ceca were not infected, nevertheless harbored a large number of worms.

In the absence of any specific action of the drug either on the organism of blackhead in the tissues or on the intestinal worm, *Heterakis papillosa*, explanation was now sought in some simple physiological action of the drug such as the increase of secretions or peristalsis. On one occasion it was thought that the droppings of the treated turkeys were more watery than those of the controls, but continued observations showed no marked difference in this respect between the two lots. The administration of ipecac in the food had, under the conditions of the experiment, a distinct retarding effect on the growth of the turkeys, possibly because a smaller amount of the drugged food was taken. No other untoward effect was noted unless the appearance of abnormalities affecting both bones and feathers in the only turkey which recovered is to be attributed to the long course of treatment.

Sulfur Treatment.—From the results obtained with ipecac it was thought probable that other drugs having evacuant properties would have a similar action. Sulfur was chosen because it is non-toxic, can thus be administered in large amounts, and is said to increase peristalsis. The number of turkeys available at this time was small.

Experiment 5.—Four turkeys of the lot of forty hatched on July 14, 1921, and five hatched on August 14 were employed. On September 1 these turkeys, now 48 and 17 days old respectively, were distributed in four cages so that some of either age could be furnished sulfur daily in their food, while the others would serve as controls. From the beginning of the experiment dirt from a hen yard was scattered in all the cages and used to contaminate the food. The younger of the turkeys treated each received one teaspoonful of precipitated sulfur twice a day, one-half being added to the sour milk, one-half to the dry mash, while the older ones each received two teaspoonfuls twice a day, similarly added to the dry mash and the sour milk. Later on the two groups that were being fed sulfur were combined in one cage and the controls in another. It was now no longer possible to estimate the dosage for each turkey but an abundance of sulfur was always available in both milk and dry mash. Sulfur, since it is a practically tasteless powder, is readily taken with the dry mash but tends to float on a fluid like milk.

The results obtained are given in Table III.

It is quite apparent that sulfur may be administered in large amounts to turkeys without untoward effect. The droppings, es-

pecially those of the cylindrical type from the small intestine, may assume a dull clayey appearance. No diarrhea was noted.

Although the number of turkeys employed was small, the results are suggestive in that with one exception the treated turkeys survived longer than the controls. It was necessary to kill two of the smaller turkeys (one control and one sulfur-treated turkey) early in the ex-

TABLE III.

Group.	Turkey No.	Treatment.	Death.	Anatomical findings.	<i>Heterakis</i> .
1 Controls.			1921		
	67*	From Sept. 1, 1921, dirt from hen yard placed in cage and also in food.	Sept. 29. D.	Typical blackhead.	47
	68*		" 26. "	" "	350 (esti-
	71†		" 20. K. (weak legs).	(Sept. 15. Operation on crop.)	mated).
	72†		Sept. 24. D.	Typical blackhead. " "	45+ 6 (1 macerated).
2 Sulfur-treated.	69*	From Sept. 1, 1921, dirt from hen yard placed in cage and also in food. Sulfur in daily ration.	Sept. 25. D.	Typical blackhead. (Sept. 9. Operation on crop.)	28
	70*		Oct. 11. "	Typical blackhead.	13+
	73†		Sept. 20. K. (weak legs).	" "	14+
	74†		Oct. 3. K.	" "	9
	75†		" 3. D.	" "	16

* Hatched July 14, 1921.

† Hatched Aug. 14, 1921.

periment (September 20), on account of helplessness resulting from faulty development. Both of these showed lesions in ceca and liver.

In general, *Heterakis* was found in the control turkeys in greater numbers than in the sulfur-fed turkeys. In the control turkey showing only six worms, the finding of a barely recognizable macerated *Heterakis* in the putrefying material from the occluded cecum suggests that worms are destroyed in the decomposition that takes place when these organs become extensively diseased.

DISCUSSION.

Since Graybill and Smith⁴ have called attention to *Heterakis papillosa* as a possible factor in the transmission of blackhead, it appears to be a matter of primary importance to establish the part played by this worm in the natural incidence of this disease. In a previous paper² statistical data were discussed, showing that no great preponderance of *Heterakis* had been found in infected as compared with normal turkeys. The opinion was also expressed that invasion of the mucosa might be effected by pathological conditions arising from other causes as well as from the presence of *Heterakis*. Reliance on the post-mortem count of *Heterakis* may, however, not be justified in all cases, for it is probable that many worms are destroyed when the cecum becomes extensively diseased, as already mentioned. It is also notable that in cases in which only one cecum is diseased, there are usually fewer worms in the abnormal than in the normal cecum.

The susceptibility of the turkey, however, is evidently so great that infection may occur with very few and possibly with no *Heterakis* present. A half grown turkey recently killed showed characteristic blackhead with involvement of one cecum and the liver. The diseased cecum showed a localized lesion not over 2 cm. in extent, while the other appeared quite normal. Only one immature *Heterakis* was found on careful search of both ceca.

Evidence of the occurrence of pathological conditions in the ceca independently of *Heterakis* and unassociated with blackhead infection has already been pointed out in the cores which sometimes form in these organs at an early age without any blackhead infection. Such cores were recently found in the ceca of two turkeys 5 and 8 days old, respectively, that had been kept in a clean compartment and fed sterilized food. In these also there was no evidence of blackhead.

Finally, our previous suggestion that blackhead may occur spontaneously in the absence of *Heterakis* is to a certain extent supported by the experimental production of the common type of the disease by feeding liver lesions from acute cases. Not only was *Heterakis* presumably absent from the material fed, but it could not be demonstrated post mortem. Young turkeys under natural conditions, however, would not have access to the virus in tissues.

⁴ Graybill, H. W., and Smith, T., *J. Exp. Med.*, 1920, **xxxi**, 647.

As to the sources of the virus in the natural transmission of blackhead, we have now experimental evidence suggesting that it may be present in the eggs of *Heterakis*, although this has not as yet been confirmed by morphological evidence. In the present investigation it was found possible to produce blackhead by feeding ripe *Heterakis* ova that had been subjected to 1.5 per cent nitric acid sufficiently long (3 days) to render the material bacteriologically sterile. It is hardly probable that the virus would survive under such conditions unless protected by the impervious membranes of the *Heterakis* ovum, for all our studies indicate that *Histomonas* is a relatively non-resistant organism. The second possibility is that the virus is present in the turkey from the time of hatching, awaiting only some exciting factor to cause it to invade the tissue. The microscopic study of tissues of many young turkeys has never revealed *Histomonas* except in blackhead infection, in which it is rarely found outside the living tissues. Furthermore, all isolated control turkeys, even those showing evidence of pathological conditions, *i.e.* cecal cores, failed to develop blackhead.

That it is only necessary to feed acid-treated *Heterakis* ova to young turkeys kept in strict isolation to produce the disease suggests that the virus is transmitted from bird to bird in the ovum of *Heterakis*. Although infection was obtained in 100 per cent of the ova-fed turkeys in certain of the experiments already outlined, postmortem examination of a large number of turkeys has already shown that the introduction of a small number of the worms may not always produce the disease. Again the disease may occur in association with very few worms. Such irregularity with respect to infection might be accounted for by the absence of *Histomonas* from a certain proportion of *Heterakis* eggs but, without morphological demonstration, this assumption is unwarranted.

There is considerable variation in the interval between the ingestion of ova and the onset of the disease. Whether this is due to the absence of *Histomonas* for a time or to conditions that prevent immediate invasion of the tissues is not known. It is also wholly problematical whether this parasite is able to persist and multiply in the cecal contents before invading the tissues. If *Histomonas* were actually introduced into the cecal mucosa by the worm larvæ, it would be difficult to account for such irregularity in the onset of infection as may, for

example, be seen in Experiment 3. In this experiment, although a number of the turkeys when killed relatively early (after 8 to 11 days) showed numerous larvæ, only one was diseased and in this case the infection was quite advanced. The efficacy of this *Heterakis* material was proved by the fact that two other turkeys fed with a portion which had also been treated with dilute nitric acid developed the disease. As no symptoms appeared in these turkeys for a period of 3 weeks, it is probable that they did not become immediately infected.

Graybill and Smith⁴ have considered *Heterakis papillosa* as a preliminary agent in that, by injury to the cecal mucosa, it may serve to lower the resistance of the turkey preparatory to the invasion of the protozoon. While this might not appear improbable, the presence of the protozoon in the ceca prior to the ingestion of *Heterakis* ova has not been demonstrated. Furthermore, little is known as to the invasive ability of *Histomonas*, although we have shown that the injection of material containing large numbers of this organism directly into the lumen of the cecum may fail to produce infection. While it is possible that the degree of the injury and the extent of the infection may depend on the dosage of *Heterakis* ova, it is known that blackhead may in some instances follow the ingestion of only a small number of these eggs. We have also found that infection may occasionally be produced, in the absence of *Heterakis* and its injurious effects, by merely feeding liver lesions containing large numbers of the protozoon. That injury of the cecal mucosa will of itself lead to blackhead infection is not borne out by the facts. Cores associated with inflammation in the ceca of young turkeys kept isolated and on a diet of sterilized food furnish an example of injury to these organs in the absence of blackhead infection. The results of the present experiments would indicate that *Histomonas* is not present in newly hatched turkeys but is acquired naturally by them as the result of the ingestion from contaminated soil of ripe *Heterakis* ova containing this protozoon. It may also be introduced experimentally by feeding fresh liver lesions. *Heterakis*, aside from any injurious effect that it may have on the cecal mucosa, would in this case be an important distributing agent of the protozoon of blackhead. However, the microscopic examination of large numbers of *Heterakis* eggs at all stages of development has thus far failed to reveal any inclusion resembling *Histomonas*.

The administration of drugs for the prevention or cure of blackhead has not been successful as employed in the present experiments. The attempt was made to obtain the maximum effect of the drugs by adding large doses to the daily food ration.

Powdered ipecac appears to have no deleterious effect on the blackhead organism in the tissues for it does not prevent the development of blackhead resulting from inoculation nor does it prevent the fatal termination of this form of the disease. One would not therefore expect it to have curative properties when used in natural blackhead. When administered prophylactically it has a definite effect in delaying the onset of the disease. This effect is not the result of any injurious action on *Heterakis*, for this worm was found in great numbers in the ipecac-treated turkeys. Ipecac served only temporarily to prevent blackhead infection, in that the treated turkeys eventually became infected and only one of the ten recovered. The claims of Wegeforth⁵ with respect to both the prophylactic and curative properties of ipecac are thus disproved by the present investigation. Since this drug showed no evident effect on either blackhead virus or on *Heterakis*, it was thought that some physiological action such as the stimulation of secretions and peristalsis might account for its tendency to delay invasion. Accordingly sulfur, on account of its mild evacuant properties, was administered to turkeys which were at the same time exposed to infection.

Sulfur in large doses appears also to retard infection, although the number of turkeys employed was not sufficiently large to furnish conclusive results. Sulfur may be slightly unfavorable to *Heterakis*, for in general the sulfur-treated turkeys showed fewer of these worms than the control birds.

The failure of *Chaparro amargosa*, an efficient remedy for amebic dysentery in the human being, to prevent blackhead in turkeys has been reported in a previous paper.⁵ Since the blackhead parasite proves to be of the nature of a flagellate rather than an ameba, it is not remarkable that it does not respond to drugs having amebicidal properties. However, the failure of tartar emetic and of quinine to alter the course of blackhead is no less marked,⁶ although the value of these drugs in certain protozoan infections is well established.

⁵ Tyzzer, E. E., *J. Med. Research*, 1919-20, xli, 211.

⁶ Tyzzer, E. E., and Fabyan, M., *J. Infect. Dis.*, 1920, xxvii, 207.

At present the most hopeful mode of attacking blackhead appears to be through measures tending to eliminate *Heterakis*. Such measures may be directed towards the isolation of the turkey or towards the destruction of the worm. It is possible that vermifuges administered at short intervals may prevent blackhead infection. However, it is not, at present, known how soon after the introduction of *Heterakis* the tissues are invaded by the blackhead parasite. Invasion may occur very early in some cases, for extensive infiltration of the tissues has been found in a very young turkey 8 days after feeding *Heterakis* material. It is safe to assume that infection occurred in this case at least as early as the 4th or 5th day. The interval between the ingestion of *Heterakis* ova and the invasion of the tissues by the parasite of blackhead may therefore be very brief, in fact it is conceivable that infection may follow immediately in some cases. Whether the administration of vermifuges at intervals will either prevent or lessen the incidence of blackhead in young turkeys must be determined by further experimentation. For the present it appears to be the safer course to rely on isolation for the prevention of the introduction of *Heterakis* to young turkeys.

The successful employment in the present investigation of soil from a hen yard for the purpose of producing blackhead furnishes additional experimental evidence as to a common source of this infection. The agencies here used for the experimental production of blackhead are present and operative in most farmyards. While it is already well established that blackhead occurs in chickens, little is known concerning its prevalence among them. During the present season typical blackhead lesions of the liver as well as of the ceca have been found in young chickens raised on ground previously occupied by mature poultry. It is probable that blackhead tends to run a mild course in young chickens, as already indicated by the experiments of Smith and Graybill,⁷ and on this account is often not recognized. That the disease of the chicken and the turkey are identical, as indicated by the transmission of blackhead to turkeys by *Heterakis* material obtained from hens, is further shown by the production of typical blackhead in the turkey through the inoculation of the latter with the liver lesions from a case of blackhead in a chicken.

⁷ Smith, T., and Graybill, H. W., *J. Exp. Med.*, 1920, xxxii, 143.

Blackhead was inoculated into nine turkeys during the course of the present season and three of these that developed active lesions completely recovered. Subsequent repeated subcutaneous inoculations of active virus were without effect. That this immunity may, however, be of short duration is indicated by the fact that, kept on infested ground, one of the above turkeys apparently passed through a mild attack of blackhead about 3 months later. We have already reported the occurrence of the disease in older breeding turkeys, male and female, and also in a hen over 2 years old. Recently an acute process was found in the cecum of a 3 year old male turkey which had always been confined with other turkeys in a small yard, where every external condition necessary for infection seemed to be present. It is evident that turkeys may have attacks of more or less severity from time to time. Occasional cases of the disease are also found in old fowls that had presumably been exposed to the infection throughout life.

The experiments conducted in the present investigation have again shown that newly hatched turkeys do well in close confinement, and notwithstanding the sterilization of the food they may develop normally for at least a month. Young turkeys infected with blackhead evidently resist the disease longer when confined than when wandering free, possibly through the elimination of the fatigue factor.

Previous conclusions as to the flagellate nature of *Histomonas meleagridis* are confirmed by observations made in the course of the present investigation. By the strict isolation of turkeys and by furnishing them sterilized food, it has been possible to keep them free from all protozoan contamination for a period of 4 weeks. Blackhead has been produced in such turkeys by feeding them *Heterakis* ova that had been treated in dilute nitric acid with the object of destroying all organisms external to the egg membrane. Under such conditions *Histomonas* was found in flagellate form in the ceca late in the disease. The flagellum of this organism was also observed in motion in incubated material from acute liver lesions. The morphological details of the flagellated forms will be considered in a subsequent paper.

CONCLUSIONS.

That the ovum of *Heterakis papillosa* is an important source of the virus in the natural transmission of blackhead in turkeys is suggested by experimental evidence obtained in the present investigation. Confirmatory morphological evidence has not thus far been obtained to establish definitely that this worm serves as the invertebrate host of *Histomonas meleagridis*.

It is possible occasionally to produce blackhead in turkeys by feeding large amounts of the virus alone, as obtained in liver lesions. This artificial procedure is, however, not duplicated in nature since the lesions of the disease are not eaten by young turkeys.

The intentional contamination of the food of young turkeys with dirt taken from hen yards has in the present experiments invariably produced blackhead. The conditions provided are essentially those that prevail in many farmyards.

The identity of blackhead infection in common fowls with that of turkeys is indicated by the production of the typical disease in a turkey by the inoculation of liver lesions obtained from a diseased chicken.

It is not known whether variation in the period between the ingestion of *Heterakis* ova and the onset of the disease is dependent on the absence of the virus for a time or on the absence of the conditions necessary for invasion.

Under experimental conditions a large proportion of the *Heterakis* ova fail, at least when fed by hand, to hatch out in the intestine, but, passing through, may later be taken up with contaminated food. This is shown by the occurrence in the ceca of larvæ in various stages of development long after the *Heterakis* material is fed.

Ipecac, like other drugs previously tested, is found to be of no practical value in the control of blackhead. It has no deleterious effect on *Histomonas* in the tissues and does not prevent the development of *Heterakis*. Its daily administration to the limit of tolerance fails to prevent infection or a fatal outcome in the naturally acquired disease.

The fact that ipecac may delay the onset of the infection may perhaps be due to some physiological property of this drug. Sulfur administered daily in large amount likewise, possibly on account of its evacuant action, tends to delay infection.

Recovery from inoculated blackhead is associated with a degree of immunity. That this is not permanent is indicated by the appearance of symptoms of blackhead several months after recovery from the inoculated disease. The fact that blackhead sometimes appears in mature turkeys that have been kept constantly on infested ground suggests that these birds may suffer from repeated attacks.

Observations made during the course of the present investigations confirm previous conclusions as to the flagellate character of *Histomonas meleagridis*.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VIII. FURTHER OBSERVATIONS ON THE CULTURAL AND MORPHOLOGICAL CHARACTERS OF *BACTERIUM PNEUMOSINTES*.

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PLATE 67.

(Received for publication, February 13, 1922.)

In an earlier paper of this series¹ *Bacterium pneumosintes*, derived from the nasopharyngeal washings of patients in the early hours of acute epidemic influenza, was described as a minute bacilloid body of regular form, with a length about two to three times its breadth, measuring 0.15 to 0.3 micron in the long axis. It was stated that although longer individuals were seen occasionally, "the organisms showed little tendency to pleomorphism and were characterized by uniformity in size and shape."

At that time, *Bacterium pneumosintes* had been cultivated only in a tissue medium composed of human ascitic fluid and fresh rabbit kidney, sometimes with the addition of beef infusion broth and nutrientagar. The combination of ascitic fluid and fresh kidney tissue has remained the medium of choice for the maintenance of the cultures, because in it they require transfer only at long intervals and they retain their morphological and cultural characteristics through many generations. But during cultivation over a period of 1 to 3 years the three strains² at present available have become saprophytic, so that at present they are cultivable anaerobically in a variety of media which are less difficult to prepare. Coincident with this adaptation to a new environment, certain variations in morphology and a loss of pathogenicity for rabbits have been observed. It is the

¹ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

² These strains were derived from Cases 16, 17, and 26 (Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125).

purpose of this paper to describe the cultural and morphological characteristics of *Bacterium pneumosintes* after prolonged artificial cultivation. The three strains have behaved in an identical manner, and a common description will suffice.

Methods of Cultivation.

Even after cultivation for 3 years *in vitro* *Bacterium pneumosintes* fails to grow in autoclaved media without the nutritive or growth-stimulating factors found in fresh tissue or body fluids. But the ascitic fluid of the original medium may be replaced by peptone broth; and fresh defibrinated blood, vegetable tissue,³ or the products of bacterial metabolism may be substituted for rabbit kidney. The addition of dextrose (1 per cent) to the medium hastens the establishment of anaerobic conditions, and, judging from the rapidity and density of the growth, increases its nutrient value.

The basis of the media now in use is a 1 per cent peptone beef infusion broth, with sodium chloride 0.5 per cent, titrated to pH = 7.4 on the Sørensen scale. According to the object for which the culture is prepared, this broth is enriched with fresh blood, animal or vegetable tissue, or by the growth of other organisms, as will be described. Solid and semisolid media are made by the addition of nutrient agar in the proper proportion.

When a large inoculum from an enriched medium is added to dextrose broth alone, *Bacterium pneumosintes* grows in the primary, but not in subsequent transplants, showing that the initial growth is dependent upon factors carried over from the tissue medium. Thjötta and Avery⁴ have shown how minute is the quantity of the growth-stimulating factors required in the case of Pfeiffer's bacillus. Their V factor in dilutions of 10^{-3} and their X factor in dilutions of 10^{-6} suffice to promote growth. With *Bacterium pneumosintes* the

³ Dr. O. T. Avery and Dr. H. J. Morgan, at the Hospital of The Rockefeller Institute, found that *Bacterium pneumosintes* grows readily in a simple infusion broth containing a fragment of fresh vegetable tissue (potato, turnip, parsnip, etc.) and kindly permit us to report their observation in advance of their own publication. They described this medium in the *Proceedings of the Society for Experimental Biology and Medicine*, 1921, xix, 113.

⁴ Thjötta, T., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 97.

essential growth-stimulating factors appear to be of the same order of efficacy. For example, 20 mg. of fresh rabbit kidney supports growth in 5 cc. of medium. The faint haze of *Bacillus coli* which develops in the 1st hour after inoculation makes dextrose broth a highly favorable medium for *Bacterium pneumosintes*. This interesting symbiotic relationship will be discussed in a subsequent section.

Cultural Characteristics.

Bacterium pneumosintes was described as an obligate anaerobe and has maintained that character. We have successfully cultivated the organism only under a vaseline seal or in the depths of the medium in the presence of active reducing agents such as dextrose, fresh animal or vegetable tissue, or aerobic organisms, or in a strictly anaerobic jar. Under such conditions, under a vaseline seal for example, *Bacterium pneumosintes* grows in enriched dextrose broth in a diffuse cloud throughout the fluid medium. In previously incubated tubes, in which anaerobic conditions are already established, this cloud becomes visible within a few (8 to 16) hours after inoculation and reaches its greatest density in 3 to 5 days, when the culture is opaque by reflected light and shows a smoky translucency by transmitted light. Growth is then checked by acid production. The cloud is too finely divided to show a bacterial shimmer, and remains in suspension for days, gradually settling in an even, amorphous, cream-colored layer in the bottom of the tube. Spontaneous flocculation has not been observed.

In the depths of solid media submicroscopic colonies develop as tiny gray specks, which, under the high power of the microscope, are found to be dense irregular masses of bacteria with a fringe of single organisms.

Dextrose is split by *Bacterium pneumosintes* with acid formation but without gas production. In dextrose broth the limiting hydrogen ion concentration is the same for all three strains; namely, 5.2 to 5.3 on the Sørensen scale. After growth has apparently ceased, however, the organisms remain viable for several days in the acid medium.

Recently the simple, safe, and efficient anaerobic jar described by Brown⁵ has furnished us with the conditions necessary for the

⁵ Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.

development of surface colonies of *Bacterium pneumosintes*. The jar is a modification of those devised by McIntosh and Fildes⁶ and by Smillie,⁷ and utilizes the catalytic activity of palladinized asbestos heated by a resistance coil in a wire-screened chamber (the principle of the Davy safety lamp) for the union of the oxygen with hydrogen. In this jar we have used plates and slants of nutrient agar, made with beef infusion broth without dextrose, enriched with 5 per cent of fresh defibrinated rabbit blood.

After incubation for 7 to 10 days blood agar plates sown with *Bacterium pneumosintes* show many very minute colonies, almost submicroscopic in size, which are round, raised, and convex, with an entire edge and a colorless translucency. No characteristic structure has been observed, and the growth does not discolor or precipitate the medium. On account of their minute size the colonies are usually discrete, even when close together, but they coalesce to form raised plaques of confluent growth in the most crowded areas.

These colonies interest us as the first example of which we are aware of surface colony formation by a filter-passing obligate anaerobe. The plates also give us a ready means of purifying contaminated cultures, and are useful for the demonstration of organisms in sparse growths of early generations in the ascitic fluid-kidney medium in which the microscopic observation of *Bacterium pneumosintes* is difficult on account of its minute size and the presence of stained protein precipitate.

Morphology.

The substitution of dextrose-peptone broth for ascitic fluid or serum in the medium results in a considerable change in the morphology of *Bacterium pneumosintes*. The dextrose, by the prompt establishment of anaerobic conditions and by its nutritive value to the organisms, supports a much more luxuriant growth than is found in ascitic fluid media. The bacteria are found in diplo form, or in chains of several members, and many of the individual organisms have increased in length so as to be obviously bacillary. They are plump rods with rather pointed ends, which give them a spindle

⁶ McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

⁷ Smillie, W. G., *J. Exp. Med.*, 1917, xxvi, 59.

shape. Stains color them deeply only in the middle and fade out towards the ends. In chains the spaces between the members are sharply demarcated. More variation occurs in length than in thickness. Forms from 0.5 to 1.0 micron long are not uncommon in cultures which show the characteristic minute forms also. Figs. 1 and 2 show the relative size and shape of *Bacterium pneumosintes* in a collodion sac dialysate of ascitic fluid and in dextrose broth respectively. A strain which has grown as fusiform bacilli in dextrose broth for several generations reverts to the original minute form on cultivation in a dialysate of the original ascitic fluid medium (Fig. 3). Aside from the differences in length and the greater tendency to chain formation, no irregularities in morphology have been noted in comparison with the early generations.

In films from surface colonies grown for 7 days on rabbit blood agar in an anaerobic jar, most of the organisms are of the minute form, but here also longer individuals are found.

As stated previously, *Bacterium pneumosintes* decolorizes by Gram's method. The organisms stain with the usual basic dyes. They are not motile. Neither capsules nor flagella have been demonstrated.

Serological Reactions.

The fusiform, bacillary forms of *Bacterium pneumosintes* grown anaerobically in enriched dextrose broth media and on blood agar plates have been tested at intervals with the immune rabbit sera produced a year ago with cultures of the organism grown in the collodion sac dialysate of an ascitic fluid-rabbit kidney medium.⁸ All three strains are promptly agglutinated by these sera, and, on the other hand, they show no tendency to spontaneous flocculation or to agglutination in normal serum. Their genetic relationship to the original minute forms of *Bacterium pneumosintes* is without question, and is further evidenced by their strictly anaerobic character and their reversion to the minute forms on transfer to the original medium.

Symbiosis.

Mention has already been made of the symbiotic development of *Bacterium pneumosintes* with *Bacillus pfeifferi*, the pneumococcus,

⁸ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.

Streptococcus hæmolyticus and *viridans*, and staphylococci, in cultures accidentally contaminated with these organisms.¹ In a number of instances *Bacterium pneumosintes* was recovered from these mixed cultures by filtration. Since the medium then in use was sufficient for the independent growth of any of these organisms, the coincident development of *Bacterium pneumosintes* and the contaminating organism did not give evidence of a nutritive interchange between them.

More recently, in cultures intentionally inoculated with a strain of *Bacillus mesentericus*, we have observed the growth of *Bacterium pneumosintes* in autoclaved dextrose broth in the absence of a fresh tissue fragment. This observation has led to the development of a simple method of anaerobic cultivation. Aerated dextrose broth is inoculated with a young culture of *Bacillus mesentericus* in which spores have not yet formed. During the first hours after inoculation the organism grows diffusely in a faint cloud without pellicle formation. The reducing activity of this obligate aerobe is shown by the decolorization of methylene blue in such a culture within a few minutes after its introduction. The culture is sealed with vaseline when inoculated, or shortly afterward and growth is inhibited by the exhaustion of free oxygen from the medium. Meanwhile the hydrogen ion concentration of the medium falls from 7.4 to 7.2–6.8. 6.8 appears to be the limiting acidity for our strain of *Bacillus mesentericus*. If such a preparation is then inoculated with *Bacterium pneumosintes*, the anaerobe grows luxuriantly and clouds the medium heavily in the course of 1 or 2 days.

This rapid growth of *Bacterium pneumosintes* carries the hydrogen ion concentration of the medium to 5.3–5.2 in the course of 3 or 4 days. Coincidentally the vegetative forms of *Bacillus mesentericus* are killed and undergo autolysis so that finally microscopic films and plate cultures may show only the anaerobic organisms. Usually, however, some few spores of *Bacillus mesentericus* are introduced in the original inoculum or are formed in the culture. These may develop subsequently and complicate results. We have therefore tested a number of other organisms for their nutritive or growth-promoting properties, combined with other characters which make them suitable for use. Of these, *Bacillus coli communis* seems well adapted to our purpose. It multiplies rapidly in plain or dextrose

broth under a vaseline seal, so that a sufficient growth is obtained within an hour or two after inoculation. During this short incubation the hydrogen ion concentration of the medium is only slightly affected. The organism may then be killed by exposure to 100°C. for 15 to 30 minutes without destroying the peculiar nutritive substances it imparts to the medium. At the same time anaerobic conditions are immediately established under the vaseline seal. *Bacillus coli* is practically non-pathogenic, and its autolyzed products are not toxic in the minute amounts contained in these cultures. The *Bacillus coli* broth is inexpensive and simple to prepare, and less liable to contamination than are unheated media containing fresh tissue or blood. It contains a minimum of foreign protein. Finally, it supports an abundant growth of *Bacterium pneumosintes* in successive generations. A large inoculum, 0.2 to 0.5 cc. of fluid culture, should be used for seeding.

Preservation of Stock Cultures.

For the maintenance of our stock cultures we have heretofore relied upon the original ascitic fluid-tissue medium, in which *Bacterium pneumosintes* after a preliminary incubation of 5 to 7 days has remained viable at room temperature for $2\frac{1}{2}$ years. Recently blood broth, without dextrose, has been used for the same purpose with encouraging results. The tests are incomplete and the limit of viability in this medium is not yet known, but *Bacterium pneumosintes* established in artificial culture appears to be a resistant organism, and probably requires transfer only at long intervals. We have also employed the method described by Swift⁹ for the preservation of microorganisms by freezing and drying *in vacuo*. *Bacterium pneumosintes* withstands this process. The dried cultures are viable for at least 2 months.

In conclusion, we have at present available a number of cultural methods and culture media suitable for a variety of purposes.

For primary isolations from filtered nasopharyngeal secretions, or from the lung tissues of affected animals, the Smith-Noguchi ascitic fluid-rabbit kidney medium probably insures success in the largest percentage of cases. It was in this medium that the original

⁹ Swift, H. F., *J. Exp. Med.*, 1921, xxxiii, 69.

isolations were made and the morphological and cultural characteristics of *Bacterium pneumosintes* were first observed.

For the maintenance of stock cultures also, the ascitic fluid-fresh tissue medium is preferred. Blood broth probably may be substituted in the case of well established cultures. *Bacterium pneumosintes* withstands freezing and drying *in vacuo* and probably is viable for long periods in the dry state.

For the demonstration of sparse growths of the microorganisms in the tissue medium, blood agar plates in an anaerobic jar have advantages over the microscope. They are useful also for the purification of contaminated cultures.

Finally, for immunization and for serological reactions requiring considerable quantities of bacteria with a minimal amount of foreign protein, dextrose broth cultures, enriched by the growth of another microorganism, furnish the most useful product. Suitable suspensions may be obtained also from blood agar plates or from growths in the dialysate liquid of collodion sacs containing the culture medium.

These methods of cultivation are being used in studies of the nasopharyngeal secretions of influenza patients obtained during the present recurrence of influenza in New York City. The results of these studies will be reported in subsequent papers of this series.

SUMMARY.

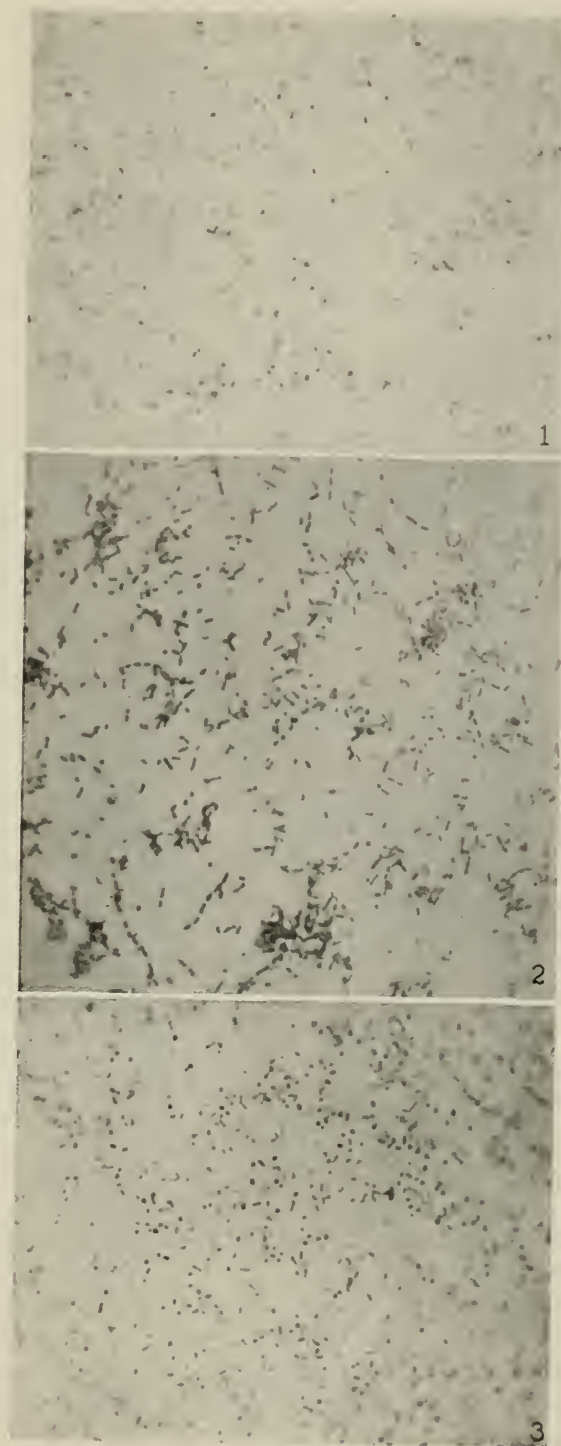
After artificial cultivation for a period of over 3 years *Bacterium pneumosintes* has maintained its original morphological and cultural characteristics, when grown in the original medium. Adaptation to a saprophytic existence has been accompanied by a loss of pathogenicity. Our strains now grow readily under strictly anaerobic conditions in a variety of media with peptone broth as a base, enriched with fresh tissue, blood, or by the growth of other bacteria. Surface colonies have been obtained on blood agar plates in an anaerobic jar. These various methods of cultivation are adapted to special purposes. In broth cultures *Bacterium pneumosintes* grows in larger forms than in the ascitic fluid-tissue medium, but the identity of the microorganisms is proved by their serological reactions and by reversion to the minute forms on transfer to the original medium.

EXPLANATION OF PLATE 67.

FIG. 1. *Bacterium pneumosintes*, Strain 16, in the twenty-fourth generation. Originally isolated March 30, 1919. Film from a collodion sac dialysate of ascitic fluid-rabbit kidney medium. $\times 1,000$.

FIG. 2. *Bacterium pneumosintes*, same strain. Film from a culture in dextrose broth and rabbit kidney medium. $\times 1,000$.

FIG. 3. *Bacterium pneumosintes*, same strain. Film from a collodion sac dialysate of ascitic fluid-rabbit kidney medium seeded from a culture in dextrose broth and rabbit kidney. Note the reversion to the original form. $\times 1,000$.



(Olitsky and Gates: Nasopharyngeal secretions from influenza. VIII.)

THE PEPTASE, LIPASE, AND INVERTASE OF HEMOLYTIC STREPTOCOCCUS.

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The study of the metabolic functions of cells and of more highly organized forms of life has shown that in many instances enzymes are responsible for the cleavage of complex molecules into their simpler components which may be utilized to support life and growth. While many of these enzymes are found in groups of plants or animals in which they accomplish the conversion of certain substances peculiar to individual species, others are more widely distributed. Among the latter group are those necessary for the disintegration of carbohydrates, fats, and proteins into the elementary molecules which are assimilated by the individual cell, so that it is not surprising that they have been found in bacterial extracts, because most bacteria are able to utilize complex organic materials for food.

It has been shown that all bacteria do not possess enzymes for the digestion of natural protein and that variations may be found in the heat resistance and action of peptolytic and sugar-splitting enzymes obtained from different bacteria. On account of these apparent differences in the identity of bacterial enzymes, the peptase, lipase, and invertase of the hemolytic streptococcus have been studied so that they may be compared with similar enzymes of other bacteria. It was hoped that further application of the methods employed would reveal differences sufficient to account for the variations in the pathogenicity of different strains of this streptococcus.

Methods.

Preparation of the Solution of Enzyme.—The enzyme solutions used in the following experiments were prepared from broth cultures

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of a beta type hemolytic streptococcus. This strain was originally obtained from a fatal case of peritonitis. Since the objects of the experiments were to perfect a method by which active solutions could be obtained and to study the physical conditions optimum for the action of these solutions on various substrates, no other strains were studied. Active hemolysis could be obtained with this streptococcus on blood agar plates and the centrifugates or filtrates of actively growing broth cultures were hemolytic. The strain fermented lactose and salicin but did not produce acid in mannitol broth. The final hydrogen ion concentration of cultures in 1 per cent dextrose broth was pH 5.0.

The streptococci from which the enzymes were extracted were obtained by the sedimentation of broth cultures after 12 to 16 hours of active growth. The broth was prepared in the usual way except that it contained 2 per cent peptone instead of the 1 per cent solution ordinarily employed in bacteriologic work. Since the tap water used in the broth contained considerable inorganic material, the first lots of media prepared precipitated heavily during sterilization. It was impossible to obtain clean bacteria when this precipitation occurred. While the broth could be filtered and autoclaved a second time, bouillon which was first titrated to pH 0.9 and boiled a few minutes at this H ion concentration, then filtered and titrated to pH 8.0 gave no further precipitate. The media prepared in this way yielded a greater quantity of bacteria than lots which were twice autoclaved. The strain employed agglutinated spontaneously in this medium so that the streptococci settled to the bottom of the flask; the supernatant fluid could then be decanted, leaving the bacterial sediment in the remaining few hundred cubic centimeters. Due to the incomplete sedimentation and the scant yield from large flasks of media, 0.1 per cent dextrose was added before sterilization. Large quantities of bacteria could then be obtained within a few hours after the flasks were seeded; the final acid concentration was between pH 6.0 and 6.5 in the dextrose broth cultures. Dextrose was used in the preparation of all enzyme solutions except in a few experiments designed to determine the effect of acid on the enzymes. It increased the growth and the acid developed was favorable for agglutination.

Large quantities of broth were required to obtain sufficient bacteria for one experiment. Usually a 6 or 12 liter flask of broth was seeded with 50 cc. of an actively growing culture. Sedimentation was complete after 12 to 16 hours incubation at 37°C., so that the supernatant medium could be siphoned off without disturbing the bacteria. The sediment was centrifuged, washed with distilled water, and transferred with a few cubic centimeters of phosphate solution ($\frac{M}{15}$, pH 7.0) to a sterile agate mortar. The mortar contained about 2 gm. of powdered glass. It was covered with thin rubber sheeting arranged so that the bacteria could be dried and ground without contamination. The streptococci were dried in a vacuum over phosphoric anhydride. When they were nearly dry the mortar was removed from the desiccator and the bacteria were ground with the occasional addition of several drops of distilled water until only a few cell bodies were sufficiently intact to retain the gentian violet in Gram-stained films. The ground material was next pipetted into a graduate and covered with a layer of toluene. This step was necessary to kill the few bacteria remaining alive after grinding. 12 hours later the bacterial suspension was pipetted from beneath the toluene and centrifuged until clear. The supernatant fluid was made up to the volume required in each experiment with $\frac{M}{15}$ phosphate solution (pH 7.0). This volume could not be more than 50 cc. without reducing the concentration of the enzyme beyond a point where decisive results were obtainable. All the glassware and solutions used in the procedure were sterile.

The enzyme solutions were opalescent at pH 7.0 but showed a flocculent precipitate when they were titrated to pH 5.0 with lactic acid. Ordinarily they varied in reaction from pH 6.8 to 7.2. Broth cultures of the final solutions were sterile. Only Gram-negative precipitate was found in the stained films of these bacterial extracts.

Preparation of the Substrate Solution.—The substrates in the majority of the experiments were peptone, cane-sugar, or esters of fatty acids. Ordinarily the solutions were prepared so that the final concentration would be 1 per cent. For the demonstration of lipolytic action the strength of the solutions of ethyl butyrate or triacetin were such that in some of the trials this final concentration was 2 per cent. Solutions or emulsions which would withstand temperatures above 100°C. were autoclaved.

Peptone solutions (either 2 or 4 per cent in distilled water) were adjusted to pH 7.0 with sodium hydroxide and sterilized by the Arnold method. The commercial bacteriologic peptone was so completely hydrolyzed that increases in amino nitrogen could not be determined after further digestion with active trypsin. The peptone¹ employed was prepared especially for these experiments. It contained 12.5 per cent nitrogen, 16 per cent of which could be determined as amino nitrogen by Van Slyke's method. After the solutions were sterilized the amino nitrogen was increased to 20 per cent of the total.

Albumin was obtained from sterile horse serum fractioned with ammonium sulfate. The precipitate was dialyzed and concentrated in a vacuum with sterile precautions. The concentration of the concentrated solution was determined by the Kjeldahl method and then adjusted to 4 per cent. This 4 per cent solution was heated at 56°C. for 1 hour. The casein was dissolved in a neutral phosphate solution and heated for 1 hour on 3 successive days in the Arnold sterilizer. Sterile horse serum was inactivated at 56°C. for 1 hour. 4 per cent solutions of the carbohydrates in $\frac{M}{200}$ phosphate mixture were sterilized in the autoclave. Cane-sugar was autoclaved at pH 7.2, dextrose at 6.8, and starch at 7.0.

Chemical Methods.—H ion concentrations were determined colorimetrically with mixtures of sodium citrate and hydrochloric acid or of phosphates prepared according to Sørensen (1). The readings were made by the comparator method with the Clark and Lubs (2) series of indicators.

Except in instances in which the macro Kjeldahl method is specified the total nitrogen and non-protein nitrogen were determined by the micro method of Folin and Wu (3). Trichloroacetic acid was used as a precipitant in the non-protein nitrogen determinations.

The amino nitrogen estimations were made with the micro apparatus described by Van Slyke (4). Solutions of alanine and leucine were first analyzed and the results were within 0.5 per cent of the theoretical values. The peptone substrates were not precipitated before analysis.

¹ This peptone was prepared by Fairchild Bros. and Foster, New York.

The percentage of inversion of the cane-sugar was determined by the colorimetric method of Folin and Wu (5). Preliminary precipitation of the small amount of protein in the mixtures of cane-sugar and enzyme was found to be unnecessary. The results have been expressed as milligrams of dextrose per 100 cc. of final solution.

The action of the lipase was determined by the method recently employed by Avery and Cullen (6). These results have been expressed in terms of cubic centimeters of $\frac{N}{50}$ alkali required to adjust the reaction of the active to that of the inactive enzyme tubes. They are based on 100 cc. amounts of substrate.

Bacteriologic Methods.—Aside from the toluene used for the sterilization of the enzyme solutions no antiseptics were required. In the preparation of the enzyme very little toluene was apparently carried over when the bacterial emulsion was pipetted into the centrifuge tubes because the digestion flasks were occasionally contaminated. Broth cultures of these flasks were made before chemical analysis; no experiments have been included in which the most rigid tests did not show sterility throughout. The purity of the cultures from which the enzymes were obtained was proven on blood agar plates.

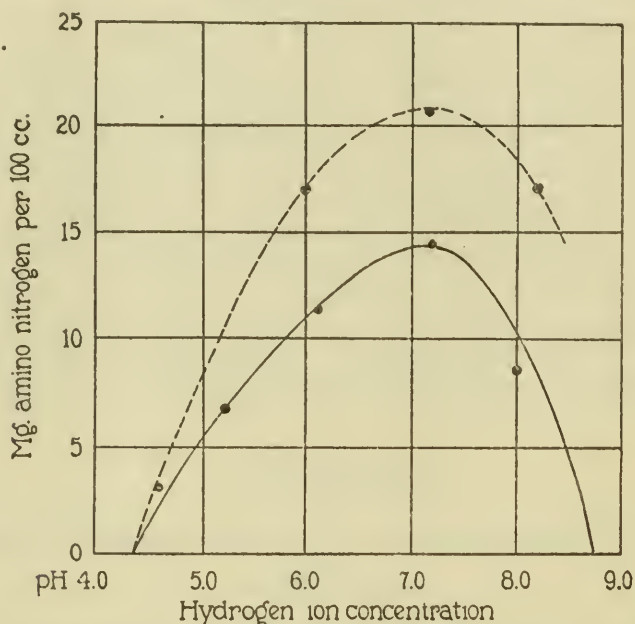
EXPERIMENTAL.

The Effect of Acid Concentration on the Action of the Peptase, Invertase, and Lipase.

Enzyme action is known to occur with the proper substrate under certain physical conditions which vary according to the nature of the enzyme. The range of acid concentration through which the reaction occurs and the optimum acid concentration are important in deciding the type of enzyme. Fuhrmann (7) noted that in a general way proteolytic bacterial enzymes responded to alkali and acid in a manner very similar to pancreatic trypsin. More recently Avery and Cullen (8) have shown that the peptase of pneumococcus has an optimum range of pH 7.0 to 7.8. This, together with the percentage of splitting obtained, led these authors to suspect that the enzyme was of an erepsin-like nature. They found likewise that the lipase (6) and invertase (9) of pneumococcus exhibited ranges similar to that of the peptonase.

Experiments 1 and 2. The Effect of Acid Concentration on the Splitting of Peptone.—Enzyme obtained from streptococci grown in 0.1 per cent dextrose broth was tested with a sterile solution of peptone as substrate. 10 cc. of the solution were pipetted into sterile 20 cc. volumetric flasks and sufficient HCl or NaOH was added to adjust the H ion concentration to the desired pH; 5 cc. of enzyme solution were added to each flask and the volume was brought up to 20 cc. with $\frac{M}{15}$ phosphate or citrate mixture of the pH desired.

Amino nitrogen determinations were done after digestion at 37°C. for 48 hours. Duplicate flasks prepared with boiled enzyme served as controls for each of the digestions. Flasks prepared without peptone served to control any increase in amino nitrogen due to the digestion of the protein in the bacterial extract. The



TEXT-FIG. 1. The effect of acid concentration on the peptone-splitting action of the enzyme solutions (Tables I and II).

object of the experiments was to determine the effect of acid concentration on the enzyme action within a sufficiently wide range to aid in establishing the identity of the enzyme. The final concentration of the peptone in the flasks was 1 per cent. Cultures made from the flasks immediately preceding analysis were sterile. The results are given in Tables I and II.

Experiments 3 and 4. The Effect of Acid Concentration on the Inversion of Cane-Sugar.—The details of these experiments are similar to those of Experiments 1 and 2 except that a solution of saccharose was used for substrate. A 4 per cent solution of cane-sugar was autoclaved in $\frac{M}{250}$ phosphate mixture at pH 7.2; 5 cc. were pipetted into each 20 cc. flask so that the final concentration of the sugar was 1 per cent. The enzyme solution was divided into equal portions, one of which was boiled for 20 minutes. Duplicate flasks were prepared at the

TABLE I.

The Effect of Acid Concentration on the Splitting of Peptone.

Flask No.	Final acid concentration.		Amino nitrogen per 100 cc. of solution.		
	Active.	Inactive.	Active.	Inactive.	Increase.
	<i>pH</i>	<i>pH</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1, 1a	8.2	8.1	41.5	24.4	17.1
2, 2a	7.2	7.2	45.6	25.1	20.5
3, 3a	5.9	6.0	40.8	24.0	16.8
4, 4a	4.6	4.6	27.9	24.9	3.0
5, 5a*	8.0	8.0	3.3	3.6	0.0

* Flasks containing boiled and unboiled enzyme without substrate to control the autolysis of the protein in the enzyme solution.

TABLE II.

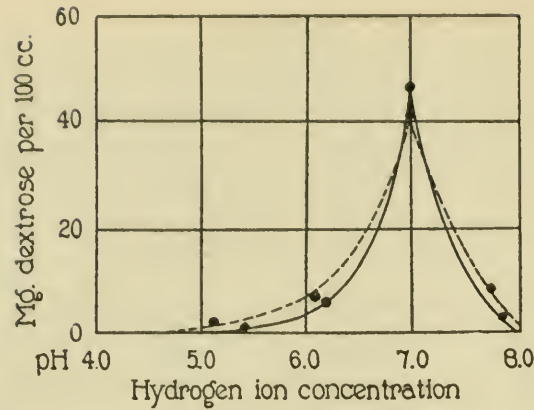
The Effect of a Greater Acid and Alkaline Range on the Splitting of Peptone.

Flask No.	Final acid concentration.		Amino nitrogen per 100 cc. of solution.		
	Active.	Inactive.	Active.	Inactive.	Increase.
	<i>pH</i>	<i>pH</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1, 1a	8.8	8.8	27.7	24.3	3.4
2, 2a	8.0	8.0	33.0	24.5	8.5
3, 3a	7.2	7.1	38.5	24.3	14.2
4, 4a	6.1	6.1	35.6	24.3	11.3
5, 5a	5.2	5.2	30.0	23.2	6.8
6, 6a	3.9	3.9	22.7	22.7	0.0
7, 7a	3.2	3.2	28.6	28.9	0.0
8, 8a*	7.0	7.1	3.5	3.1	0.4
9, 9a†	7.0	7.0	26.6	26.6	0.0

* These flasks contained active and inactive enzyme without peptone.

† After the supernatant fluid was removed from the centrifuge tubes in the preparation of the enzyme solution the sediment consisting of ground streptococci was washed once and divided in half. One half was boiled. Digestion tubes were prepared with this boiled and unboiled sediment and peptone.

various acid concentrations with 5 cc. of active and 5 cc. of inactive enzyme. The invert sugar was determined as dextrose by the micro method of Folin and Wu after 48 hours of incubation. Cultures from the flasks were sterile. The invert sugar has been expressed as milligrams of dextrose per 100 cc. of final solution. Two experiments have been included in Table III on account of the peculiar type of curve obtained.



TEXT-FIG. 2. The effect of acid concentration on the inversion of saccharose (Table III).

TABLE III.

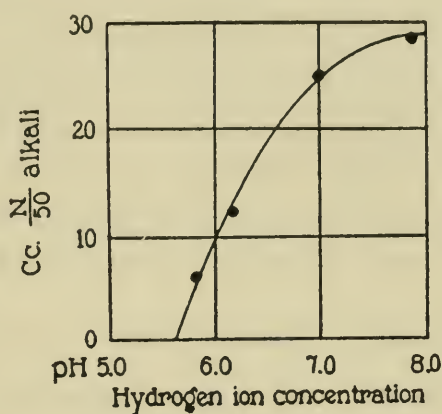
The Effect of Acid Concentration on the Inversion of Saccharose.

Flask No.	Acid concentration.			Dextrose per 100 cc. of solution.		
	Initial.	Final.		Active.	Inactive.	Increase.
	Active.	Active.	Inactive.			
	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1, 1a	7.8	7.8	7.8	9.5	7.5	2.0
2, 2a	7.0	7.0	7.0	53.2	7.5	45.7
3, 3a	6.2	6.2	6.2	12.1	7.4	4.7
4, 4a	5.4	5.4	5.4	8.1	7.5	0.6
1		7.7	*	15.9	*	8.6
2		7.0		49.0		41.7
3		6.1		12.8		5.5
4, 4a		5.1	5.1	10.0	7.3	2.7

* In order to concentrate the enzyme in this experiment with the hope that greater inversion might be obtained, controls with boiled enzyme were omitted from the series with the exception of the control on Flask 4. This was chosen to rule out any possibility of interpreting as a result of enzyme action, increased amounts of dextrose which might occur purely from acid hydrolysis after long incubation at pH 5.1.

Experiment 5. The Effect of Acid Concentration on the Hydrolysis of Ethyl Butyrate.—The details of this experiment are similar to those of the preceding four experiments except that ethyl butyrate was used as substrate. 5 cc. of a 4 per cent emulsion of ethyl butyrate sterilized in the Arnold sterilizer were pipetted into each of eight flasks so that the concentration was 1 per cent after the volumes were made to 20 cc. Four of the flasks were prepared with boiled

and four with unboiled enzyme solution and the volume was made up with $\frac{M}{5}$ phosphate of the desired pH. After 48 hours of incubation the active enzyme mixtures were adjusted to the pH of the similar control containing the boiled enzyme. The results have been expressed in Table IV in cubic centimeters of alkali required to adjust the pH of 100 cc. of the solution after hydrolysis to that of the control flask containing the boiled enzyme.



TEXT-FIG. 3. The effect of acid concentration on the hydrolysis of ethyl butyrate (Table IV).

TABLE IV.

The Effect of Acid Concentration on the Hydrolysis of Ethyl Butyrate.

Flask No.	Acid concentration.				Amount of N/50 NaOH required in titration. cc.
	Inactive.		Active.		
	Initial.	Final.	Initial.	Final.	
	<i>pH</i>	<i>pH</i>	<i>pH</i>	<i>pH</i>	
1, 1a	7.8	7.8	7.8	7.1	29.0
2, 2a	7.0	7.0	7.0	6.6	25.0
3, 3a	6.2	6.2	6.2	5.8	12.0
4, 4a	5.8	5.8	5.8	5.6	6.0

The effect of acid concentration on the action of these enzymes is similar to the effect observed with the enzymes of other bacteria. In this respect they are nearly identical with the endoenzymes obtained by Avery and Cullen (6, 8, 9) from pneumococci, both with regard to the range of acidity through which they are active and the optimum acid concentration.

The peptolytic enzyme (Text-fig. 1) is not active below pH 4.4 and has an optimum action at about pH 7.2. The acid limit for

the inversion of cane-sugar was about pH 5.0 and the optimum action occurred at pH 7.0. According to Text-fig. 2 the curves showing the effect of acid on inversion differ from those for peptolytic and lipolytic action. Below pH 7.0 the type of curve obtained would appear to indicate that the optimum range was narrower for the invertase than for the other enzymes; while this part of the curve is established on three determined points, the curve above pH 7.0 in Text-fig. 2 is hypothetical because there are only two determinations within this range. It is possible that with more active preparations of invertase the curve would be similar to those obtained for the lipase and the peptase. The lipase of this streptococcus has an optimum reaction more alkaline than either of the other enzymes studied (Text-fig. 3).

The Effect of Heat on the Action of the Peptase, Invertase, and Lipase.

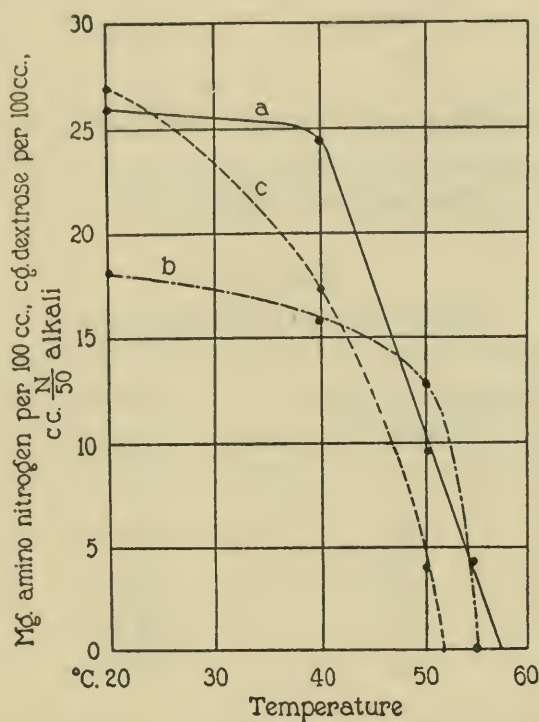
Susceptibility to heat is one of the characteristics of enzyme solutions. The temperature which destroys the enzyme varies with the source and type of enzyme and the conditions under which the heat is applied. Dry enzyme or enzyme in solution with substrate may resist temperatures which are destructive to simple aqueous solutions. The enzyme solutions in the following experiments were extracted from the streptococci without the addition of materials (with the exception of the toluene used in the procedure) foreign to bacterial growth and with a minimum amount of protein present. Neutrality was maintained during the extraction and heating. For this reason it is not possible to compare the results with those of previous workers too closely. The digestions were carried out at approximately the optimum acid concentration.

Experiment 6. The Effect of Heat on the Peptolytic Enzyme.—The enzyme solution was prepared as in the preceding experiments. It was divided among several thin walled tubes which were plunged into water of the desired temperatures for 10 minutes and afterward cooled quickly in ice water; 5 cc. of each of the heated portions were pipetted into flasks with 5 cc. of 4 per cent peptone solution. The volume of each flask was made up to 20 cc. with $\frac{M}{15}$ phosphate mixture (pH 7.0). Amino nitrogen determinations were made after incubation at 37°C. for 48 hours (Table V). The flasks were sterile after incubation. The initial and final pH of each flask was 7.0.

Experiment 7. The Effect of Heat on the Invertase.—This experiment is similar in every respect to Experiment 6 except that saccharose was used as the substrate.

Flask 1 contained enzyme which had been boiled 20 minutes. The substrate, saccharose, was autoclaved in 4 per cent solution at pH 7.2. The volume of each digestion was 20 cc. so that the final concentration was 1 per cent. The invert sugar was determined by the micro method after 48 hours incubation (Table VI). The initial pH and the final pH of each flask were 7.0. Cultures from the flasks were sterile.

Experiment 8. The Effect of Heat on the Lipase.—The procedure was similar to that in the preceding experiments. Ethyl butyrate served as substrate. A 4 per cent emulsion was sterilized in the Arnold sterilizer; 5 cc. were then pipetted into the digestion flasks with 5 cc. of enzyme solution which had been subjected



TEXT-FIG. 4. The effect of heat on the solutions of (a) peptase, (b) lipase, and (c) invertase (Tables V, VI, and VII).

to temperatures of 20°, 40°, 50°, and 55°C. for 10 minutes. The enzyme in Flask 1 was boiled 20 minutes. The volume of each flask was made up to 20 cc. with $\frac{M}{15}$ phosphate solution. The titrations were made with $\frac{N}{50}$ NaOH. The results are expressed in cubic centimeters of alkali required to adjust the reaction of 100 cc. of digest to that of the control flask prepared with boiled enzyme (Table VII).

The results obtained in the preceding experiments on the effect of exposing the enzyme solutions to heat have been summarized in Text-fig. 4. In order to correlate the various terms in which the results have been expressed considerable interpolation has been nec-

TABLE V.

The Effect of Heat on the Peptolytic Enzyme.

Flask No.	Temperature at which enzyme was heated for 10 min.	Amino nitrogen per 100 cc. of solution.	
		Analysis.	Increase.
	°C.	mg.	mg.
1	100*	26.9	
2	60	26.5	0.0
3	55	31.3	4.4†
4	50	36.2	9.3
5	40	51.3	24.4
6	20	52.9	26.0

* Flask 1 was prepared with enzyme which was boiled 20 minutes.

† Cultures of the streptococcus in broth were not viable after they were heated at 55°C. for 10 minutes. They were not killed at 50°C.

TABLE VI.

The Effect of Heat on the Invertase.

Flask No.	Temperature at which enzyme was heated for 10 min.	Dextrose per 100 cc. of solution.	
		Analysis.	Increase.
	°C.	mg.	mg.
1	100	7.2	
2	55	7.0	0.0
3	50	48.2	41.0
4	40	178.7	171.5
5	20	277.7	270.5

TABLE VII.

The Effect of Heat on the Lipase.

Flask No.	Temperature at which enzyme was heated for 10 min.	Acid concentration.		Amount of N/50 NaOH required in titration.
		Initial.	Final.	
	°C.	pH	pH	cc.
1	100	7.7	7.7	
2	55	7.7	7.7	0.0
3	50	7.7	7.4	13.0
4	40	7.7	7.3	16.0
5	20	7.7	7.3	18.0

essary to bring all the curves within a similar range. The temperatures required to destroy these enzymes were between 50° and 60°C. and corresponded closely with the thermal death-point of the streptococcus from which they were obtained. The results suggest that the death of the bacteria is due to the destruction of the enzymes. Such a conclusion, however, would not be in agreement with the previous literature. Repeated references are made to bacterial enzymes which are destroyed only by temperatures far in excess of that required to kill the bacteria from which they were obtained. Avery and Cullen found this to be true for the pneumococcus.

The Effect of Exposure to Acid.

The length of time during which solutions of enzyme remain active depends largely on the physical conditions under which they are preserved. In the preceding experiments temperatures above 55°C. destroyed the enzymes when they were in solution at reactions optimum for their action. There was slight deterioration even at a temperature as low as 40°. In view of this sensitiveness to heat in neutral solution, a series of experiments was devised to discover the effects of weak acid. The enzyme was exposed to lactic acid by titrating to pH 5.0, incubating at 37°C. for 6 hours, and finally adjusting the solution to the optimum acidity. Lactic acid was chosen because it is one of the principal fermentation products of dextrose in broth cultures of streptococcus (10).

Experiment 9. The Effect of Exposure to Acid.—The bacterial sediment from 12 liters of 2 per cent peptone broth was extracted in the usual manner. Dextrose was not added to this broth because it was desirable to avoid exposing the bacteria to acid during their growth. The enzyme solution was divided into three equal portions to which 10 drops of methyl red and phenolsulfonephthalein were added. One lot was boiled for 20 minutes; the second portion was adjusted to pH 5.0 with lactic acid; the third served for a control. All three were incubated at 37°C. for 6 hours. The acid tube was titrated to pH 7.0 (the acid concentration which had been maintained in the other tubes during incubation) with NaOH at the end of this period. All three tubes were then made up to equal volumes with phosphate mixture (pH 7.0, $\frac{M}{15}$). The action of these lots of enzyme was observed with substrates of peptone, cane-sugar, and triacetin. The triacetin (8 per cent emulsion) was autoclaved at pH 7.0; the other substrates were prepared as in the preceding experiments. 5 cc. of substrate and enzyme solution were pipetted into

digestion flasks and made up to 20 cc. volume with phosphate solution. The analyses were made after incubation at 37°C. Cultures from these flasks showed no growth. The results are shown in Table VIII.

TABLE VIII.
The Effect of Exposure to Acid.
(a) *Peptolytic Enzyme.*

Flask No.	Treatment of enzyme.	Acid concentration.		Results per 100 cc. of solution.	
		Initial.	Final.	Amino nitrogen.	Increase.
		<i>pH</i>	<i>pH</i>	<i>mg.</i>	<i>mg.</i>
1	Boiled 20 min.		7.1	33.7	
2	Incubated at 37°C. for 6 hrs. at pH 5.0 and adjusted to pH 7.0.		7.1	49.8	16.1
3	Normal active enzyme.		7.1	63.9	30.2

(b) *Invertase.*

Flask No.	Treatment of enzyme.	Acid concentration.		Results per 100 cc of solution.	
		Initial.	Final.	Dextrose.	Increase.
		<i>pH</i>	<i>pH</i>	<i>mg.</i>	<i>mg.</i>
4	Boiled 20 min.	7.0	7.0	6.3	
5	Incubated for 6 hrs. at pH 5.0 and adjusted to pH 7.0.	7.1	7.1	6.4	0.1
6	Normal active enzyme.	7.1	7.1	44.0	37.7

(c) *Lipase.*

Flask No.	Treatment of enzyme.	Acid concentration.		Amount of N/50 NaOH required in titration.
		Initial.	Final.	
		<i>pH</i>	<i>pH</i>	<i>cc.</i>
7	Boiled 20 min.	7.0	6.8	
8	Incubated 6 hrs. at 37°C. at pH 5.0 and adjusted to pH 7.0.	7.0	6.8	0*
9	Normal active enzyme.	7.0	6.2	94.0

* On account of the partial hydrolysis of the inactive triacetin control the flasks were adjusted to pH 6.8. Controls without enzyme demonstrated that after triacetin had been autoclaved the reaction was more acid after 48 hours by pH 0.1 to 0.3.

Experiment 10. The Effect of Exposing Weak Solutions of Peptolytic Enzyme to Acid.—The preceding experiment was repeated with a weak solution of enzyme. The procedure was otherwise similar. The acid-treated enzyme was tested only with a substrate of peptone. The solution with which Experiment 9 was carried out yielded a heavy precipitate when it was acidified with acetic acid. The enzyme in this experiment did not show a precipitate but merely opalescence after the addition of weak acetic acid. The results of the experiment are shown in Table IX.

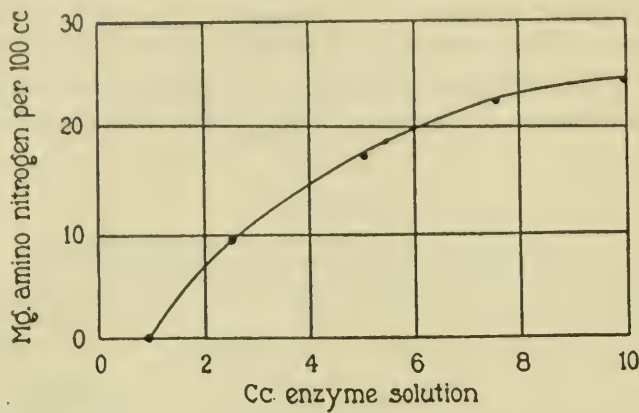
TABLE IX.
The Effect of Acid on Weak Solutions.

Flask No.	Treatment of enzyme.	Final acid concentration.	Amino nitrogen per 100 cc. of solution.	
			Analysis.	Increase.
		pH	mg.	mg.
1	Boiled 20 min.	6.9	33.5	
2	Incubated 6 hrs. at pH 5.0 and adjusted to pH 7.0.	7.0	37.4	3.9
3	Normal active enzyme.	7.0	48.0	14.5

These experiments demonstrate that two of the enzymes are exceedingly susceptible to acid and that the third, the peptase, is partially destroyed at pH 5.0. It is difficult to account for the selective action of acid aside from the protective action which the protein in the solution might have for the peptase. Analyses show that the protein was far in excess of the percentage of reducing substances found in these solutions and for that reason it is logical to suppose that it protected the peptolytic enzyme. The invertase was probably more easily destroyed by acid in the absence of substrate or digestion products. The percentage of fat in the solutions was not estimated. In the second of these experiments the enzyme solution was less concentrated and contained but little protein; the deterioration of the enzyme was considerably greater. There is, of course, no objection to interpreting these results as due to actual differences in the character of the enzymes, since it was impossible to obtain purer solutions for further experiments.

Experiment 11. The Effect of Enzyme Concentration on Peptolytic Digestion.—The enzyme and substrate were prepared as in the preceding experiments. Flasks were prepared with 5 cc. of 4 per cent peptone solution and increasing

amounts of enzyme. The volumes were made to 20 cc. with $\frac{M}{15}$ phosphate mixture. Analyses were made after 48 hours of incubation at 37°C. (Table X and Text-fig. 5).



TEXT-FIG. 5. The effect of concentration of enzyme on the splitting of peptone (Table X).

TABLE X.
The Effect of Concentration of Enzyme on the Splitting of Peptone.

Flask No.	Amount of enzyme.	Final acid concentration.		Amino nitrogen per 100 cc. of solution.		
		Active.	Inactive.	Active.	Inactive.	Increase.
	cc.	pH	pH	mg.	mg.	mg.
1	10.0	7.2	7.2	52.1	28.3	23.8
2	7.5	7.1	7.2	50.9	28.7	22.2
3	5.0	7.2	7.2	45.5	28.3	17.2
4	2.5	7.1	7.1	38.0	28.4	9.6
5	1.0	7.2	7.2	28.4	28.7	0.0

Table X shows that the weaker concentrations of enzyme are more effective in digesting peptone than are more concentrated solutions. Bayliss (11) has shown that the time element is important in concentration studies but concludes that weight for weight, regardless of the type of velocity curve which digestion with enzymes may show, higher concentrations are more effective than the lower ones. In this respect, it is apparent that this peptase follows the general law of enzymes.

Experiment 12. The Proteolytic Action of the Enzyme Solutions.—The solution of enzyme was prepared in the usual manner from 6 liters of 0.1 per cent dextrose broth culture of streptococci. The serum albumin was obtained by fractioning

sterile horse serum with ammonium sulfate, then dialyzing, and concentrating the dialyzed solution in vacuum; this procedure was carried out without bacterial contamination. The solution was heated to 56°C. for 1 hour before it was used. The casein solution was sterilized in the Arnold sterilizer. The horse serum was inactivated at 56°C. The solutions were prepared in 4 per cent concentrations; the peptone and casein were weighed but the albumin and horse serum were diluted until they contained 4 per cent protein reckoned on the basis of the total Kjeldahl nitrogen. This, of course, introduced a slight error in the protein concentration of the diluted horse serum. The solutions were adjusted to pH 7.0 with sterile acid or alkali.

5 cc. of enzyme solution, 5 cc. of substrate, and 10 cc. of phosphate solution were pipetted into sterile flasks. These mixtures were incubated at 37°C. for 3 days and analyzed by the micro Kjeldahl method after precipitation with trichloroacetic acid (Table XI). Duplicate and triplicate precipitations with 10 per cent trichloroacetic acid with solutions of various proteins demonstrated that the precipitation was equal and constant.

TABLE XI.

The Proteolytic Action of the Enzyme Solutions.

Flask No.	Substrate.	Final acid concentration.		Non-protein nitrogen per 100 cc. of solution.		
		Active.	Inactive.	Active.	Inactive.	Increase.
		<i>pH</i>	<i>pH</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	Casein.	6.9	6.9	25.6	12.6	13.0
2	Albumin.	6.9	6.9	15.5	15.1	0.4
3	Serum.	7.0	7.0	21.1	19.3	1.8
4	Peptone.*	6.9	6.9	63.8	28.0	35.8

* The peptone digestion was included as a control on the activity of the enzyme solution. These results are expressed in milligrams of amino nitrogen per 100 cc. of solution.

The results obtained in the preceding experiment indicate that the enzymes have but slight ability to bring about the digestion of higher proteins. The increase in non-protein nitrogen in the albumin-enzyme mixture is so small that it may have resulted either from autolysis of proteins in the enzyme solution or from an experimental error in the determinations. A series of determinations with solutions of enzyme showed increases of 0.1 to 0.2 mg. of non-protein nitrogen per 100 cc. of solution after active bacterial extracts were allowed to autolyze for several days. This increase, however, is so slight that

it need hardly be considered. The digestion of casein is definite since the increase was 13 mg. in 100 cc. of digest; this indicates that over 8 per cent of the protein was attacked. The failure of the enzyme to digest serum albumin might be due either to an antienzymatic action of this protein or to an inability of the enzyme to attack it. In view of the increase in the non-protein nitrogen of the serum after digestion, an increase which may be explained by the splitting of protease normally found in serum in small amounts, it seems probable that there is little inhibition of the enzyme by the serum albumin. Such action on casein and failure to digest albumin are characteristic of the erepsin-like enzymes previously found in many plants (12). From the tables given by Avery and Cullen in their study of the proteolytic action of the pneumococcus endoenzymes it is apparent that the "peptase" which they obtained acted similarly on casein and egg albumin, since they were unable to obtain mixtures beyond that due to the autolysis of the proteins occurring in the enzyme solutions alone.

The Effect of Chloroform and Gentian Violet on the Peptolytic Enzyme.

Early in these experiments it was found necessary to employ an antiseptic in the preparation of the bacterial extracts to kill the few bacteria which escaped grinding. Since toluene was known to have a definite bactericidal action and had been used generally to preserve solutions of organic material or to prevent bacterial growth during digestion with enzymes, it was used throughout these experiments in preparing the solutions. It was felt that other antiseptics might be used which would serve a similar purpose. A series of experiments was then carried out to study the effects of chloroform and gentian violet.

Experiment 13. Comparison of the Effects of Toluene and Chloroform on the Peptolytic Enzyme.—An emulsion of the bacteria was prepared in the usual manner. After the mass was well ground half was allowed to stand with toluene and half with chloroform for 12 hours. Each half was centrifuged after that period and the supernatant solutions were pipetted from the tubes without disturbing the layers of antiseptic. The solutions were sterile. Digestion flasks were prepared with substrates of peptone. Controls containing enzyme which had been boiled 20 minutes were included in the series. After 48 hours at 37°C. the amino

nitrogen was determined for each flask (Table XII). Cultures made in broth showed no growth.

TABLE XII.
The Effect of Chloroform on the Peptolytic Enzyme.

Antiseptic.	Amino nitrogen per 100 cc. of solution.		
	Active.	Inactive.	Increase.
	mg.	mg.	mg.
Toluene.....	53.9	26.1	27.8
Chloroform.....	34.6	28.9	5.7

Experiment 14. The Inhibition of the Peptolytic Action of Enzyme Solutions by Gentian Violet.—Solutions of enzyme and peptone were prepared with toluene in the usual manner. Dilutions of gentian violet (Grübler) were made in $\frac{M}{15}$ phosphate solution (pH 7.0). · 10 cc. of each of these dilutions were pipetted into sterile flasks with 5 cc. of enzyme and 5 cc. of 4 per cent peptone solution. Amino nitrogen determinations were made after 48 hours at 37°C. (Table XIII). Controls without gentian violet were prepared with boiled and normal active enzyme.

TABLE XIII.
The Effect of Gentian Violet on the Peptolytic Enzyme.

Flask No.	Dilution of gentian violet.	Precipitate.*	Amino nitrogen per 100 cc. of solution.	
			Analysis.	Increase.
			mg.	mg.
1	1:1,000	++++	29.8	3.0
2	1:5,000	+++	37.9	11.1
3	1:10,000	++	43.8	17.0
4	1:20,000	+	43.6	16.8
5	1:50,000	+	56.8	30.0
6	1:100,000	+	57.0	30.2
7	Active normal enzyme.		56.8	30.0
8	Inactive enzyme.		26.8	

* Precipitate was formed in these flasks during incubation; the amount of precipitate has been indicated by plus signs.

Previous workers have observed that chloroform has an inhibiting action on digestion with enzyme; this has been noted frequently in the literature with respect to pancreatic trypsin. According to Fuhrmann, van Laer observed that solutions of diastase were inactive

if they were preserved with chloroform for more than 15 days. Fuhrmann (7) furthermore noted that chloroform was detrimental to bacterial enzyme action, while toluene was one of several antiseptics which might be used for the sterilization of cultures to demonstrate enzyme action. The results of Experiment 14 serve merely as confirmatory evidence of these facts. Although sterilization was accomplished with both of the antiseptics the use of chloroform resulted in considerable deterioration of the solutions.

The effects of the dilutions of gentian violet on the splitting of peptone were more surprising because Churchman (13) found that this dye had no effect on the digestion of proteins with trypsin. A very definite decrease in the peptolytic activity of these enzyme solutions was found in dilutions as high as 1:20,000. In 1:1,000 dilution the enzyme was practically inactive. Slight precipitation occurred throughout the series of flasks to which gentian violet had been added, but was most marked in dilutions more concentrated than 1:20,000. Since precipitates were not obtained with solutions of pure albumin or with the enzyme solution in similar dilutions of the dye, a series of tubes was prepared with 1 per cent of the peptone used in the experiment.

Precipitate occurred in approximately the same amounts as had been found in the enzyme-peptone mixtures. From nitrogen determinations on the supernatant fluid obtained by centrifuging these peptone mixtures it was found that nearly one-tenth of the peptone was carried out of solution in a 1:1,000 dilution. The degree of precipitation in the various dilutions corresponded roughly to the inhibition of the enzyme in similar concentrations of the dye. In dilutions of 1:50,000 and 1:100,000, which will ordinarily inhibit Gram-positive bacteria, digestion was not hindered. These observations are not sufficiently comprehensive so that one may offer an entirely satisfactory explanation for the bacteriostatic action of gentian violet. It is known that violet-positive bacteria (14) will absorb sufficient dye during growth on agar containing almost imperceptible quantities so that the growing colonies acquire a deep purple color, and that greater percentages will entirely inhibit growth. These facts suggest that its bacteriostatic effect may be due to the absorption of sufficient quantities of dye to inhibit enzyme action.

Evidently, since the trypsin digestion of proteins is not hindered by gentian violet, the inhibition is not due to a direct action on the enzyme. Furthermore, only one-tenth of the peptone was carried out of solution by the dye so it is not probable that the digestion was inhibited to any great extent by a change in the concentration of the substrate. The most plausible explanation is an absorption of the enzyme by the precipitate which might occur in Gram-positive bacteria when exposed to gentian violet as well as in the peptone-enzyme mixtures.

DISCUSSION.

The method used to extract the enzymes from the streptococci is a combination of various procedures which have been employed by previous investigators. Various innovations were introduced, such as the use of the phosphate mixtures during the grinding and sterilization of the cultures to protect the enzymes from deterioration. Further work showed that the three enzymes studied were exceedingly susceptible to heat, acid, and antiseptics, so that all the attempts made to protect them during extraction were justified. A method was desired which might be applied to other bacteria and would yield solutions of enzyme relatively free from cell debris. Although considerable protein was carried into solution the consistent results which were obtained throughout the experiments lead us to suspect that the physical conditions under which the various digestions were studied were not greatly modified by the material dissolved from the cell bodies. No attempt was made, however, to purify the solutions further.

The methods of study are similar to those previously employed by Avery and Cullen (6, 8, 9) in their work with pneumococcus, except that micro methods of analysis have been employed when possible in preference to methods requiring larger quantities of material for analysis. The enzymes resemble those which Avery and Cullen obtained from the pneumococcus, since they act through similar ranges of H ion concentration. The resemblance between the enzymes leads us to believe that similar enzymes exist in other bacteria.

The experiments on the effects of acid concentration showed that the enzymes studied were most active between pH 7.0 and 8.0. This

pH corresponds closely with the degree of alkalinity necessary for the optimum growth of streptococci. The lipase was most active at a pH more alkaline than that required for the other enzymes; this appears to be true for lipase obtained from other than bacterial sources. The acidity at which digestion might proceed varied. The lipase was found inactive at a point less acid than the final pH of cultures in dextrose broth, but the peptase, according to the curve constructed in Text-fig. 1 was active at an acid concentration slightly greater than pH 4.5. The limits of digestion in acid probably depend both on a union of substrate and enzyme and on the destruction of the enzyme itself; some of the later experiments on the effect of acid serve to elucidate this point. In Experiment 9 an acid concentration of pH 5.0 was found to be sufficient to destroy the invertase and lipase if solutions were exposed to the acid for 6 hours at a temperature of 37°C., while the peptolytic enzyme was still active after similar treatment. This suggests that the digestion obtained with the peptase in acid concentrations which completely inhibited the lipase and the invertase may be due to the resistance of that enzyme to acidities as low as pH 5.0.

Further work on physical conditions destructive to the enzymes was continued in Experiments 6, 7, and 8. A temperature of 60°C. continued for 10 minutes was found to be sufficient to destroy all three enzymes. According to the curves in Text-fig. 4, there was some deterioration even at 40°C., and the temperatures required for complete destruction were between 51° and 57°C. The streptococci from which the extracts were obtained were not viable after cultures were heated for 10 minutes at 55°C. The close agreement between these temperatures suggests that the destruction of the enzymes may be one of the factors causing the death of the organism by heat. The great number of previous observations on this point is hardly compatible with such a conclusion, since repeated observations by other investigators indicate that bacteria are ordinarily killed by temperatures which will not destroy their enzymes. Fuhrmann, however, observes that solutions of enzyme obtained from bacteria grown on media relatively free from protein are destroyed at lower temperatures than enzymes obtained from cultures on ordinary media. Other observations with enzymes from non-bacterial sources indicate that

products of digestion have a distinctly protective action. Throughout these experiments the peptase was the most stable of the enzymes. Analyses indicate that the solutions of enzyme contained considerable protein material; there was relatively little reduction of alkaline copper solutions, so we assume that there was a much smaller percentage of dextrose. It is probable that the amounts of protein and sugars in the solution account for the difference in stability of the enzymes. Such an assumption would explain the higher temperatures required to destroy the enzymes of pneumococci obtained by the complete solution of the bacterial bodies with the resulting autolysis and accumulation of digestion products.

Efforts were made to ferment dextrose by the addition of freshly ground bacteria and of yeast coenzyme to the digestion mixtures. No changes in acidity were observed after these procedures. Starch was not attacked by the enzyme solutions.

CONCLUSIONS.

1. A method has been outlined by which the enzymes of hemolytic streptococcus may be extracted with comparative ease.

2. The peptolytic enzyme is active between pH 4.4 and 8.7 with an optimum action at pH 7.2. It is destroyed in neutral phosphate solution at a temperature of 57°C. continued for 10 minutes and at pH 5.0 deteriorates slowly at 37°C. Concentration experiments with solutions of the enzyme have shown that it resembles other enzymes. It is exceedingly susceptible to chloroform and its action is inhibited by dilutions of gentian violet. Casein is attacked but serum albumin is not digested after 3 days at 37°C.

3. The invertase is active between approximately pH 5.0 and 8.0 with an optimum of pH 7.0. It is destroyed by a temperature of 52°C. continued 10 minutes at an acid concentration of pH 7.0, or after 6 hours at 37°C. at pH 5.0. At this acidity it is more susceptible to heat than the peptase.

4. The lipase is active above pH 5.6. The greatest activity was observed at pH 7.9. It is completely destroyed after heating to temperatures over 55°C. for 10 minutes and resembles the invertase in its susceptibility to acid.

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STUDIES ON EXPERIMENTAL PLETHORA IN DOGS AND RABBITS.*†

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The object of the present communication is to present the functional changes produced by repeated transfusions in the blood-making and blood-destroying apparatus and in metabolism, and also the structural changes in the viscera of dogs and rabbits. The source of an anemia that developed during the plethoric stage has also been considered. In an attempt to throw further light on the relation of the spleen to blood formation and blood destruction, we first studied the effect of splenectomy in artificial plethora, and tried to find evidence of increased enzyme action in the spleen removed at a time when blood was being destroyed in greatly increased quantities. Not only were these efforts barren of results, but it was also found that our knowledge of the changes caused by the artificial induction of plethora was in itself meager.

I.

Blood Formation and Destruction.

A few writers have studied the effect of plethora on rabbits, animals notoriously inconstant in their hemopoietic reactions. Hess (1) induced plethora in rabbits by repeated transfusions in order to study the effect on the work of the heart, and Itami (2) studied the tissue changes in these same animals. These writers also described an extremely plethoric rabbit, which, in spite of continued transfusions and of the absence of isolysins, developed a marked anemia. Robertson (3) has described more thoroughly this type of plethoric anemia and has shown

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the relationship of plethora and anemia to bone marrow activity, as indicated by the percentage of reticulated red cells. This writer found that agglutinins developed coincidentally with the anemia, but did not give quantitative details of the results. Kambe and Komiya (4) also encountered this anemia in rabbits that had been made plethoric and also demonstrated the presence of agglutinins qualitatively.

Ottenberg and Thalhimer (5) found that isohemolysins are seldom present in the blood of normal cats, but that they often appear in the blood of recipient cats after transfusions. With the development of hemolysins, the animals developed a marked anemia, from which they quickly recovered after cessation of transfusions. Ottenberg, Kaliski, and Friedman (6) demonstrated the presence of isoagglutinins and isohemolysins in dogs. These authors also showed that direct transfusion of blood that is agglutinated and laked by the recipient's blood is followed by the destruction of the transfused blood with an intense intoxication. Boycott and Douglas (7, 8) observed that dogs do not destroy cells during the days immediately following a first transfusion, but that once the animal has become accustomed to destroying blood, it can dispose of the transfused red blood cells two to eight times as fast as after the first transfusion. They therefore think that some mechanism must be developed by the animal for the rapid destruction of foreign red cells. Since Boycott and Douglas could find no evidence of isohemolysins after the transfusions, they concluded that transfused red cells were not destroyed by direct hemolysis.

Methods.

Plethora was produced in dogs by daily transfusions of 25 to 200 cc. of whole blood taken from six compatible donors in rotation. Dogs weighing from 6 to 10 kilos were used as recipients and larger animals as donors. With careful technique the jugular veins were used during the entire experiment for aspiration of blood from the donor and injection into the recipient. The donor's blood was drawn into an Erlenmeyer flask containing 2 cc. of 10 per cent sodium citrate for every 100 cc. of blood, to prevent clotting. The blood was allowed to flow into the recipient from a sterile burette by gravity. The hemoglobin and the total blood volume figures were used for estimating the degree of plethora.

Hemoglobin was determined by the Newcomer (9) method, and the vital red method of Keith, Rowntree, and Geraghty (10) was used in the estimation of the blood volume. The method of Wilbur and Addis (11) was employed for the determination of urobilin as an index of blood destruction.

Bone marrow activity was studied by estimating the percentage of reticulated or "skeined" erythrocytes. It is suggested that for the sake of brevity, the word "reticulocyte" be substituted for "reticulated erythrocyte," and the term "reticulosis" be designated to replace some such expression as "an increase in the number of reticulated cells," as is now the custom. The percentage of reticulocytes was estimated after vital staining with Grüber's brilliant cresyl blue.¹ The recipients were fed a standard diet of beef heart, cracker meal, sugar, lard, bone ash, and salt, and the donors were given table scraps. The test animals gained in weight during the experiment, while the donors became only slightly anemic with only a small rise in the percentage of reticulocytes. Removal of the spleen was performed under ether anesthesia without noteworthy loss of blood.

RESULTS.

Production of Plethora.

Seven dogs were rendered plethoric by the method described. The degree of plethora obtained did not depend on the amount of blood injected; for instance, one dog given twenty transfusions, averaging 48 cc., attained as great a rise in hemoglobin as another injected twenty-three times, each transfusion averaging 157 cc. The largest number of transfusions in one animal was 61 in 76 days; the shortest period was 15 days with eleven transfusions in a fatal case. The maximum hemoglobin obtained in these plethoric animals varied from 146 to 226 per cent (Text-figs. 1 to 4).

A peculiar result was noted in three out of four dogs (Nos. 1, 2, and 3) when transfusions were discontinued during the height of plethora; namely, a temporary rise in hemoglobin of 11, 17, and 21 per cent, occurring on the 3 following days. This was also noted by Boycott and Douglas in one of their rabbits. The drop in hemoglobin after the sudden rise was almost as striking. The fourth dog (No. 4) maintained a constant hemoglobin level for 10 days before the transfusions were stopped, and upon ceasing injections, there was a sudden drop. It is suggested that possibly some organ, such as the liver, acts as a blood storehouse, and upon cessation of transfusion, a further rise in

¹ American preparations of this stain have not been of any use in our hands.

blood count is caused by excess blood being thrown into the blood stream.

The results of our investigations have shown that the excess blood is destroyed as rapidly by the splenectomized as by the normal dogs, after ceasing the transfusions. Three dogs were made plethoric, their spleens removed, and transfusions stopped; the fourth dog used for a control was made plethoric but was not splenectomized. In all four cases, after discontinuing transfusions, the blood was destroyed with approximately equal rapidity, as indicated by hemoglobin and blood volume studies. After 4 to 6 weeks the hemoglobin had returned to normal and then a moderate anemia (hemoglobin varying from 15 to 27 per cent below normal) persisted for a few weeks, followed by a permanent return to normal. This anemia might be considered as an expression either of the time required by the bone marrow to recover from its enforced inactivity, or more probably of the organism's attempt to rid itself of its newly acquired powers of increased blood destruction.

The first few transfusions raise the hemoglobin percentage only very slightly. The reactions of animals during this time differ widely. Dog 3, for example, was transfused with 620 cc. of blood in 4 days, causing the hemoglobin to rise from the normal of 86 per cent to only 98 per cent. Another dog's (No. 2) hemoglobin increased only 9 per cent by the 3rd day after 95 cc. of blood had been transfused. The organism can apparently handle this excess blood during the first few days of transfusions without showing any signs of marked increased blood destruction, as will be shown below in the urobilin and metabolism studies.

One apparently successful attempt was made to break down an animal's blood-destroying mechanism (No. 5) by transfusing relatively massive quantities of blood (averaging 133 cc. per day). It was possible, by this means, to obtain a rise in hemoglobin from 98 per cent to 200 per cent in 11 days. With the continuance of these large daily transfusions, however, this very high blood concentration was after 8 days reduced by the organism to 150 per cent. Continued transfusions then apparently killed the animal after a second rise in hemoglobin to 226 per cent. The red cell count, at this time, was 15,500,000, as compared with the normal of 7,160,000. The cause of death of this animal will be considered later.

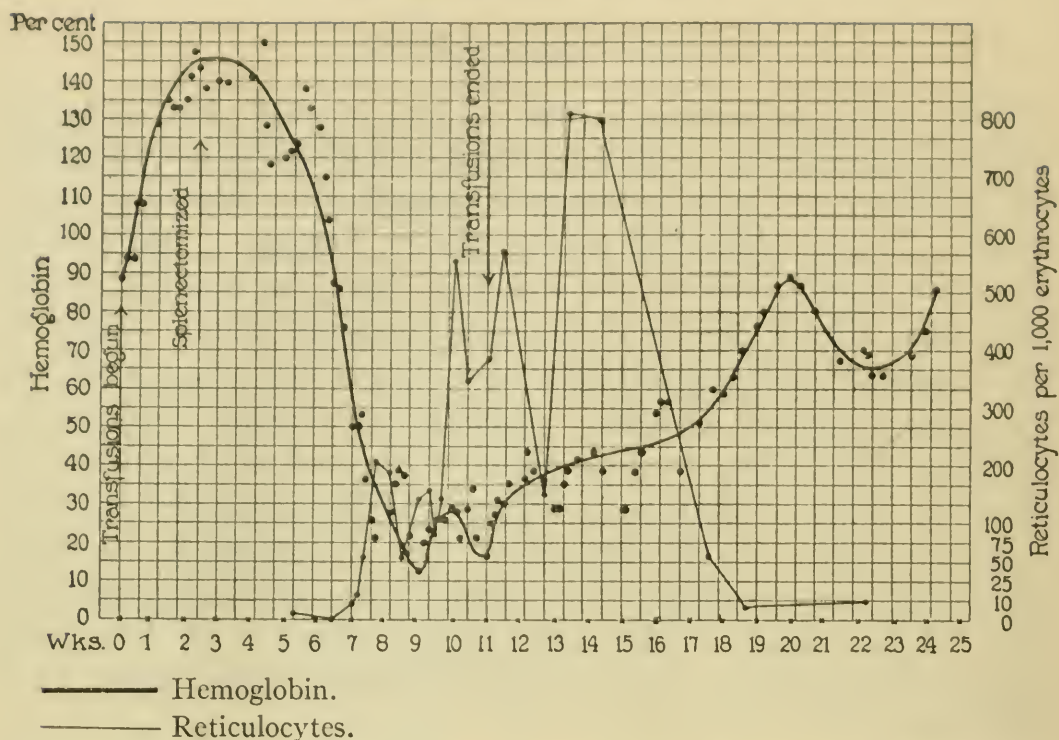
Anemia Following Plethora.

This anemia, developing in spite of and probably on account of continued transfusions, is characterized by a marked fall in hemoglobin and red blood cells with at first little reticulocyte evidence of regeneration. In one splenectomized dog (No. 6, Text-fig. 1), the anemia developed rapidly until, at the end of 15 days, the hemoglobin had fallen from 138 per cent to 21 per cent; there was then a temporary rise for several days, followed by a fall to 13 per cent, when it reached the point of greatest severity. Although transfusions were continued daily at this time, the hemoglobin at the end of 13 days was 17 per cent. The injections were stopped and the hemoglobin returned to normal slowly. In another animal (No. 7, Text-fig. 3), in which transfusions were continued after splenectomy, the hemoglobin fell from 185 per cent to 67 per cent during a period of 18 days; with continued transfusions, however, the hemoglobin rose again to a high figure.

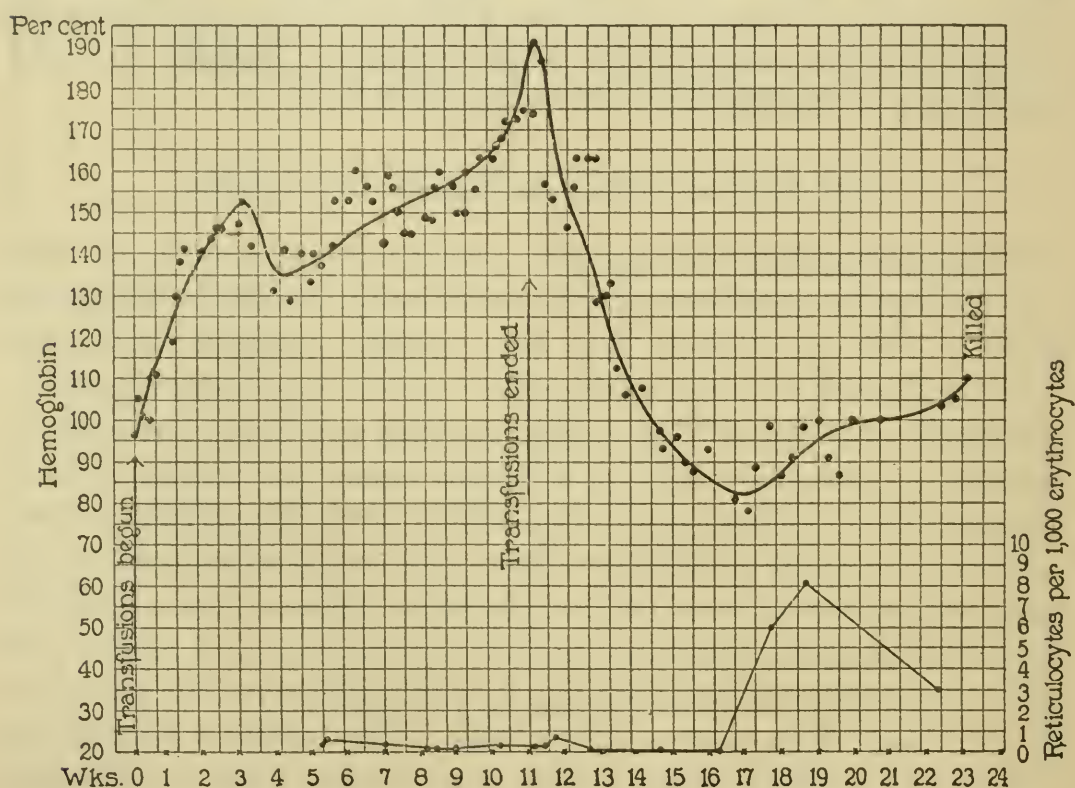
The experiment with Dog 6 was controlled by transfusing equal amounts of blood from the same donors into a normal dog (No. 4). This animal developed an artificial plethora (which persisted until transfusions were discontinued) without anemia (Text-fig. 2). The significance of this disparity will be discussed later.

Bone Marrow Activity in Plethora and Anemia.

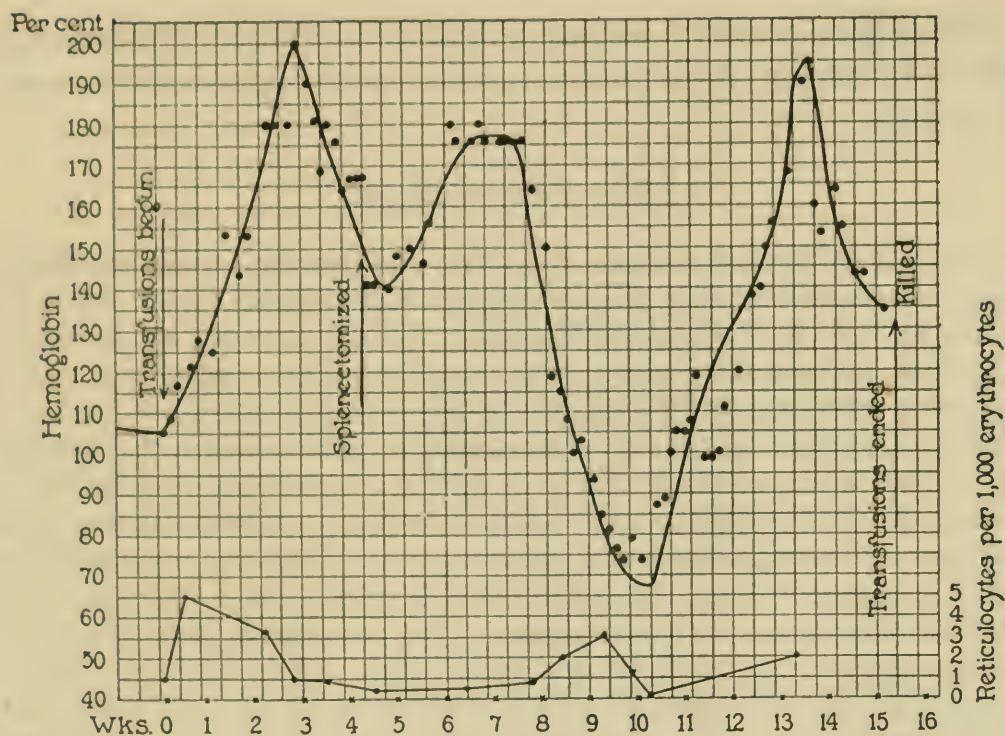
Our experiments with dogs have confirmed Robertson's (3) findings in rabbits that reticulocytes practically vanished during the stage of artificial plethora, but were greatly increased during the "plethoric anemia" stage (Text-figs. 1 to 4). The value of reticulocytes as an index of bone marrow activity and blood formation is especially evident in Text-fig. 1, which shows the sudden anemia following plethora in Dog 6. The sudden fall in hemoglobin is seen to be accompanied by a slight increase in reticulocytes, which had been practically absent in the plethoric stage. This reticulosis became marked after the anemia had been in progress 2 weeks, and after transfusions had been stopped, the reticulocytes increased to the extremely high level of 81 per cent. The animal's hemoglobin and reticulocytes gradually returned to normal after transfusions



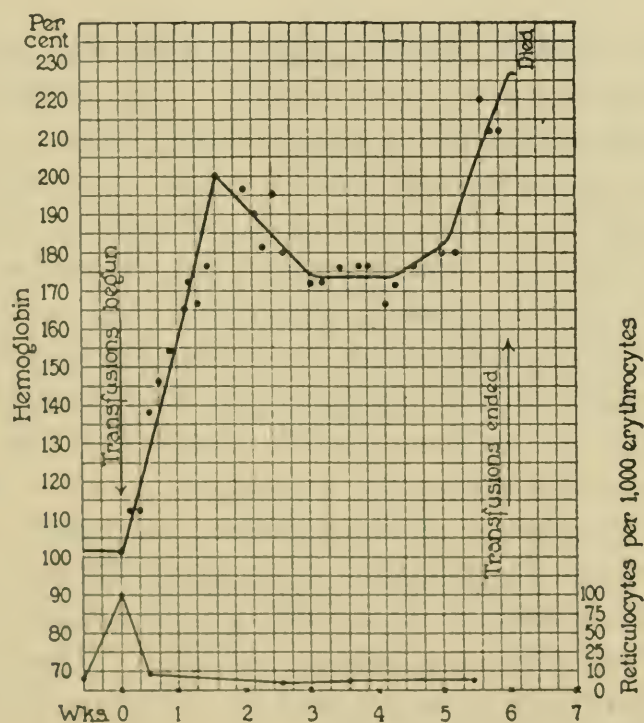
TEXT-FIG. 1. Dog 6. Reticulocyte and hemoglobin curves in experimental plethora and "plethoric anemia." The amount of blood transfused was 5,965 cc.



TEXT-FIG. 2. Dog 4. Reticulocyte and hemoglobin curves in experimental plethora. The amount of blood transfused was 6,150 cc.



TEXT-FIG. 3. Dog 7. Reticulocyte and hemoglobin curves in experimental plethoria and "plethoric anemia." The amount of blood transfused was 10,450 cc.



TEXT-FIG. 4. Dog 5. Reticulocyte and hemoglobin curves in fatal experimental plethoria. The amount of blood transfused was 5,000 cc.

had been discontinued. In Dog 7, in which a slight anemia developed after splenectomy, the slight increase in reticulocytes should be noted in passing.

Blood Volume Studies.

The vital red method of Keith, Rowntree, and Geraghty gives, according to Smith, Arnold, and Whipple (12), an accurate index of plasma volume, but is less reliable for red cell volume determinations. One can, however, obtain comparable results when working on the same animal for a long period of time. Keith, Rowntree, and Geraghty (10) observed in many anemic patients an increase of plasma volume and found that in polycythemia the plasma volume may be either low or high—in the latter case true plethora may be said to exist. Bock (13), on the other hand, studying the blood volume in pernicious anemia, has shown that as long as the hemoglobin remains above 30 per cent, there is a remarkable constancy of plasma volume. In patients with lower hemoglobin, there is a corresponding reduction of the plasma volume. The variations in blood volume are, for the most part, due to changes in red cell content. Denny (14) also finds that the plasma volume is essentially normal in pernicious anemia, and a decrease in total volume is due to loss of cell mass. The constancy of the plasma volume is also shown by Peters and Rubnitz's (15) report that the plasma nitrogen has no tendency to decrease in anemia.

In four dogs studied, the total blood volume was, as might be expected, greatly increased above normal in the plethoric stage and decreased below normal in the anemic stage (Tables I to IV). With the exception of one animal (No. 5), which died from the induced plethora, the plasma volume showed surprisingly few changes, either in anemia or plethora. The changes in red cell volume were chiefly responsible for the variations in total blood volume, so that the plethora might properly be termed "acythemetic plethora."

It is interesting to note, in agreement with Bock's and Denny's findings, that in the extreme anemia obtained in Dog 6, the total red cell volume was estimated, once at 55 cc. and 5 days later at 37 cc., while the plasma volume remained practically normal. The total blood volume dropped from a normal of 980 cc. to a minimum of 512 cc. The anemia in Dog 7 was manifested by a change from the normal blood volume of 1,000 cc. to 794 cc. The plasma volume in this dog during the anemic period was identical with the normal, with a diminished red cell volume.

With extreme plethora (No. 5) there was a marked diminution in plasma volume, together with an increased red cell volume. This diminution of plasma volume is present to a slight extent in all cases of extreme plethora during the early stages of transfusions. There is then manifested a tendency for the plasma volume to return to the original level.

Urobilin Excretion.

The amount of urobilinogen and urobilin in the urine and stools is now generally recognized to be a fairly accurate index of the degree of blood destruction in the body (Wilbur and Addis (11) and Robertson (16)). As the experimental animals were on a constant diet throughout the experiments, Whipple's (17) demonstration that bile pigment elimination can be greatly changed by modification of diet does not apply to these experiments.

Urobilin was found to be present in the urine in small amounts only during periods of marked blood destruction. Urobilin determinations were made on the feces of three dogs (Nos. 4, 6, and 7), the first two of these having received equal amounts of blood from the same donors. Dog 4 reached a maximum urobilin excretion of 8,000 units at the same time that the plethora reached its maximum. At this point transfusions were stopped, and both blood count and urobilin excretion returned gradually to normal. Dog 6 showed a maximum output of 14,400 units at the height of the "plethoric anemia." This high urobilin output continued for about 4 weeks. When transfusions ended, and the dog's hemoglobin began to rise, accompanied by a simultaneous reticulosis, the urobilin excretion was greatly diminished. Dog 7 also showed a greatly increased urobilin elimination but press of work did not permit many determinations at this time. (Tables I to III.)

Blood Counts.

Hemoglobin and erythrocyte determinations have already been considered. Leucocytes, estimated during the course of the experiments, were considerably increased, chiefly due to an increase of polymorphonuclear leucocytes; but changes were so inconstant and unexplainable that they will not be further considered. Fragility

tests made on several animals during the course of the experiments showed an increase in both the maximum and minimum resistance, both during the plethoric and anemic stages, but these were not

TABLE I.

Blood Changes in Experimental Plethora and "Plethoric Anemia."
Dog 6.

Length of time after first transfusion.	Hemo- globin.	Red blood cells.	Plasma volume.	Red cell volume.	Total blood volume.	Urobilin units.	Remarks.
days	per cent	millions	cc.	cc.	cc.		
(Normal.)	87		545	435	980	400	Mar. 9, 1920. Transfusions begun.
5	116		580	640	1,220	600	
8	128		645	925	1,570		
14	135		515	1,095	1,610	1,920	Splenectomized.
21	140		445	865	1,310		
23	140		427	905	1,332		
35	120		512	798	1,310		
37	123		475	775	1,250		
40	133		520	780	1,300		
45	87	4.6	583	373	956	2,400	
49	50	3.5	590	175	765		
51	53	2.5	560	115	675	11,200	
53	26	1.8	607	54	661		
57	27	1.2	640	70	710		
63	13	0.65	580	55	635	14,400	
68	26	1.39	475	37	512	13,000	Injectations ended.
77	17	1.71				11,300	
78	25	1.55					
95	39	1.97	437	110	547		
102	39	2.6				3,120	
112	57	2.88	535	230	765		
122	52	3.1	550	194	744		
127	59	3.8	568	210	778	640	
158	64	3.68	568	190	758		
166	69		578	272	850		
171	87		510	300	810		
215	82	5.6					

5,965 cc. of blood transfused in 60 injections; average 99 cc. per transfusion.

sufficiently striking or constant to be given in detail. Except for the slight increase in resistance of the slightly anemic donor's cells, it is difficult to see why such a change in the fragility of the blood of

the experimental animals should have taken place during plethora. A possible explanation would be that with the increased need for

TABLE II.
Blood Changes in Experimental Plethora.
Dog 4.

Length of time after first transfusion.	Hemo- globin.	Red blood cells.	Plasma volume.	Red cell volume.	Total blood volume.	Urobilin units.	Remarks.
days	per cent	millions	cc.	cc.	cc.		
(Normal.)	105		580	448	1,028	300	Mar. 9, 1920. Transfusions begun.
7	111		540	880	1,420	1,200	
14	141					1,500	
20	146		508	966	1,474		
22	153		465	825	1,290		
33	140		460	860	1,320		
42	153		378	806	1,184		
44	160		380	810	1,190	2,340	
46	156		525	805	1,330		
49	144		562	1,000	1,562		
51	156	10.9				4,400	
53	140		535	995	1,530		
57	144	11.8				1,800	
63	156	12.8	440	935	1,375	5,400	
72	168	12.7	342	928	1,270	1,870	Injections ended.
77	174					3,230	
78	174	12.7					
80	181	13.8				8,000	
100	108	12.8	515	515	1,030		
104	93	7.4				2,400	
110	88	7.0	565	425	990		
122	88		537	253	790		
127	81	6.6	515	302	817		
132	98	7.4				2,160	
158	105	6.6	592	468	1,060		Killed.
163	110					720	

6,150 cc. of blood transfused in 59 injections; average 104 cc. per transfusion.

blood destruction, the more fragile cells from each transfusion were soon destroyed, leaving the average of those remaining higher than normal.

TABLE III.
Blood Changes in Experimental Plethora and "Plethoric Anemia."
Dog 7.

Length of time after first transfusion.	Hemo- globin.	Red blood cells.	Plasma volume.	Red cell volume.	Total blood volume.	Urobilin units.	Remarks.
<i>days</i>	<i>per cent</i>	<i>millions</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>		
(Normal.)	105	6.84	550	450	1,000	400	May 17, 1920. Transfusions begun.
5	121	7.9	612	638	1,250	900	Splenectomized.
9		9.1					
24	168	12.4	460	1,840	2,300		
30	167	12.4	392	1,298	1,690		
39	146	10.7	342	948	1,290		
44	176		432	1,168	1,600		
51	176	11.6	545	1,475	2,020		
58	117	7.4	460	500	960		
65	85	5.8	560	300	860		
71	74	5.1	555	239	794		
80	98		527	448	975	4,000	Killed.
87	138	7.5	510	622	1,132		
95	195		512	1,088	1,600		
100	155		440	935	1,375		

10,450 cc. of blood transfused in 88 injections; average 118 cc. per transfusion.

TABLE IV.
Blood Changes in Fatal Experimental Plethora.
Dog 5.

Length of time after first transfusion.	Hemo- globin.	Red blood cells.	Plasma volume.	Red cell volume.	Total blood volume.	Remarks.
<i>days</i>	<i>per cent</i>	<i>millions</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	
(Normal.)	98	7.16	364	411	775	May 25, 1920. Transfusions begun.
1	102	8.12				Death 4 days later.
2	112					
4	138	9.16				
8	172	11.2				
12	200	12.8				
18	180	14.4	325	1,295	1,620	
24	172	15.2				
30	172		174	792	966(?)	
40	220	15.5	212	1,418	1,630	

5,000 cc. of blood transfused in 39 injections; average 133 cc. per transfusion.

II.

Metabolism Studies.

Although early in our work we devoted our attention solely to urobilin excretion, it later seemed advisable to study the influence of the artificial plethora and its consequent anemia on the nitrogenous metabolism, in the presence of daily parenteral introduction of large amounts of isoprotein.

The dogs used for each experiment were placed in metabolism cages, on a constant diet of raw beef heart, cracker meal, sugar, lard, bone ash, and sodium chloride, that had a known, sufficient caloric value. Water was given *ad libitum*. After 1 or 2 weeks on this special diet, if the weight of the animal remained constant, a preliminary metabolism study was made; Dogs 4 and 6 were first studied metabolically after the transfusion experiments were well in progress. The constant diet was continued between periods of metabolism study, so that although the introduced nitrogen was not actually determined, changes in elimination can reasonably be attributed to the transfused blood. Each dog was catheterized at the end of 24 hours, with the exception of one male (No. 6) chosen before the metabolic studies were contemplated. Although this renders the 24 hour periods less precise than those of the females, the length of the series studied prevents gross error in this particular experiment, if averages of several days are considered. None of the animals showed loss of weight or other ill effects during the period of metabolic study, except one (No. 5) which on account of anorexia and diarrhea could not be included in this study.

In the analysis of the urine, the total nitrogen was determined by the Kjeldahl-Gunning method, urea by Folin's aeration method, ammonia by the procedure of Van Slyke and Cullen, creatinine and creatine by Folin's method, uric acid by the Hotchkiss-Benedict modification, and total phosphates by the uranium acetate method.

Forster (18, 19) demonstrated many years ago that whole dog blood transfused into a dog in moderate amounts had a marked stability, since he failed to obtain an appreciable rise in the nitrogen output. On the other hand, foreign serum injected in the same manner is destroyed more as though given *per os*. Tschiriew (20), confirming the experiments of Forster, found that dog blood introduced by

mouth is completely metabolized in contrast to an equal amount of "living" dog blood, which may be given intravenously with little or no increase in nitrogen excretion. From these experiments, we must conclude that transfused blood protein is not immediately destroyed and that its protein is utilized when given as a food.

The nitrogenous metabolism of Dog 7 is presented (Table V) as an example of the changes that occur before and during the plethoric and anemic stages. Although the nitrogen intake in food and blood transfusions was not determined, the results of nitrogen output are

TABLE V.
Nitrogen Metabolism in Experimental Plethora.

Length of time after first transfusion.	Weight.	Urine per day.									Feces per day.	
		Volume.	Specific gravity.	Total N.	NH ₃ N	Urea N.	Creatinine.	Creatine.	P ₂ O ₅	Uric acid.	N	
				gm.	gm.	gm.	mg.	mg.	mg.	mg.		
	kg.	cc.										
Normal (5 day period)	9.19	555	1,014	4.31	0.30	3.33	328	181	309	59	0.65	
3rd and 4th days (2 day period) . .	9.13	635	1,013	4.28	0.26	3.34	262	233	189	54		
8th to 13th day (6 day period) . .	9.37	585	1,015	4.89	0.30	3.80	291	208	255	63	0.62	
24th " 29th " (6 " ") . .	9.50	740	1,011	5.28	0.32	4.31	314	121	273	69	0.46	
Splenectomized on 30th day.												
31st to 35th day (5 day period) . .	9.14	592	1,016	6.98	0.31	5.82	338	172	377	55	0.50	
47th " 51st " (5 " ") . .	9.26	538	1,013	7.08	0.30	6.07	307	225	302	85	0.40	
58th " 63rd " (6 " ") . .	9.10	478	1,018	7.31	0.31	6.28	279	143	352	72		
66th " 70th " (5 " ") . .	9.03	400	1,017	6.07	0.31	5.11	251	117	282	59		
73rd " 81st " (9 " ") . .	9.27	502	1,014	6.33	0.28	5.17	299	143	299	85		

so striking that certain conclusions can, nevertheless, be drawn. In Dog 7 during the first 10 days of the experiments, 1,200 cc. of blood were transfused, representing roughly 36 gm. of nitrogen. The nitrogen output in urine and feces for this period was practically normal, indicating that the nitrogen in excess of that accounted for by the plethoric vascular system was being stored by the body. With continued transfusions, the nitrogen output rose, but never commensurately with the amount injected. With the onset of anemia, the nitrogen excretion rose to its highest point; but during the course of the anemia, there was no such marked nitrogenous excretion.

Dog 4 was first studied metabolically while the hemoglobin was returning to normal after transfusions had been stopped. The first period, during plethora, showed a high nitrogen output; when a decrease in blood volume had occurred, the nitrogen excretion was practically normal as judged by the food intake.

In Dog 6 metabolism studies were first done while transfusions were being made, during the period of anemia. A relatively high nitrogen output was found, which decreased to about normal soon after the transfusions were stopped. During the period of recovery, there was a much lower excretion of nitrogen, which appears to be lower than normal. It is problematic at what stage the excess nitrogen of transfused blood is excreted, since there is apparently no indication from these analyses that excess, or even equal amounts of nitrogen are excreted at any time except previous to sudden development of anemia. Albumin was occasionally present in the urine during extreme plethora and at the beginning of anemia, but never in quantities that would account for the nitrogen introduced.

The creatinine, creatine, and phosphate studies give no noteworthy information about the processes concerned in blood destruction. The urea excretions paralleled the total nitrogen, but the ammonia excretion was apparently not affected. The feces nitrogen was relatively constant. It was thought that the cell destruction would be so great that a study of uric acid metabolism might be of interest, even though allantoin determinations were not made. The result, however, showed no constant or marked changes.

In the urine of all animals made extremely plethoric, bile and albumin could be detected in appreciable amounts. This was also noted during the period of anemia.

III.

Postmortem Findings.

By far the most striking change in the organs of our plethoric animals is the tremendous deposition of hemosiderin in the spleen, liver, lymph nodes, and bone marrow. Its almost complete absence from other organs is strong evidence of the important part that these organs play in the storage and elimination of the products of increased

blood destruction. Its absence from the kidney accords with the absence of hematuria or hemoglobinuria, though differing from various experimental and clinical conditions in which pigment deposition has been marked in this organ.

In animals killed or dying while transfusions were being continued, there is also, as would be expected, extreme congestion of blood vessels throughout the body. This is so marked in the viscera that details of structure often are obscured, so that it is usually impossible, for instance, to determine whether given groups of cells are packed into phagocytes or into small vessels. It is safe to say, however, that pigment phagocytosis either entirely replaces or at least is far greater than erythrocyte phagocytosis in the several organs of the hemopoietic system. The engulfing of whole erythrocytes by the phagocytes of the hemopoietic system probably only occurs in acute cases of greatly increased blood destruction and then only for a short time. In the extreme type of blood destruction that follows the administration of hemolytic serum, for instance, Pearce and Austin (21) only found erythrocytes within the phagocytes for a few days in dogs, and Karsner, Amiral, and Bock (22) found in splenectomized cats that this phase was practically complete after 48 hours. Voegtlin, Hooper, and Johnson (23) have also observed it in acute T. N. T. poisoning of dogs, but here marked fragmentation of the circulating erythrocytes and in the perfusates of spleen, liver, and bone marrow was also found.

In the spleen and lymph nodes of our animals, minute granules of hemosiderin are here and there found in large numbers free in the pulp and sinuses, and even within the blood vessels. These observations tend indirectly to support Rous and Robertson's (24) view that erythrocytes are chiefly destroyed by fragmentation into hemoglobin-containing dust, which is then removed from the circulation by the phagocytes of several organs.

The sinuses of the spleen and lymph nodes are chiefly filled by myriads of endothelial phagocytes containing hemosiderin granules of varying size. They are also numerous in the splenic pulp and even scattered through the lymphoid tissue of the lymph nodes. In smaller amount hemosiderin-bearing phagocytes persist in animals that have not been transfused for many weeks whose blood picture

has returned to normal. Hemosiderin also is found extracellularly in large clumps, apparently from fusion of pigment from phagocytes.

In the liver, hemosiderin-containing cells are also prominent; from their position between the strands of liver cells many are apparently Kupffer cells, but others occurring in Glisson's capsule and in the portal vessels are obviously the usual endothelial phagocyte. Thrombosis of bile ducts and proliferation of bile capillaries and fibrous tissue are also found. The strands of liver cells are so compressed by the congested capillaries that in many places they remain atrophied after normal conditions have been restored. A similar condition is found in some lymph nodes, where the greatly distended sinuses practically fill the node so that there is very little lymphoid tissue remaining. Grossly, lymph nodes appear enlarged, reddened or reddish brown, with a purplish center. Histologically, many of these changes are seen to be due to great congestion of the blood vessels with accumulation of the above mentioned phagocytes. In other instances, however, erythrocytes are found, sometimes in great quantities both in the central and peripheral sinuses, mingled with the usual content of endothelial, lymphoid, and plasma cells. No evidences of hemorrhage are to be found, so that the appearance of a hemolymph node is strongly suggested. In the absence of special injections, this point cannot be definitely settled, but the impression remains strong that under the need for greatly increased blood destruction, structures resembling hemolymph nodes are developed.

The bone marrow exhibits not only the same pigment deposition, but also considerable cell hyperplasia. This was not found by Itami in plethoric animals and certainly the blood picture during life points to lessened erythropoiesis. In Boycott and Douglas' animals, on the other hand, Price-Jones found a definite cellular increase, though not necessarily of cells concerned in blood formation. If this increase were only of the leucocytic series it might be correlated with the leucocytosis constantly found; but erythroblasts and unidentified cells were also increased in number. The only observations we can offer in explanation are the lack of specificity of bone marrow response, to which attention has been called elsewhere, and the impossibility of determining in a cellular bone marrow just which cells are concerned with blood cell formation and which with blood destruction. Cer-

tainly the accumulation of hemosiderin, both intra- and extracellular, and the prominence of multinuclear giant cells in the bone marrows of recovered animals point to the importance of the bone marrow as an active agent in increased blood destruction. In the recovered bone marrow, not only were multinuclear giant cells unusually numerous, but so called mature elements (polymorphonuclears and normoblasts) were considerably more prominent than in animals killed during transfusion.

In the other organs examined no noteworthy lesions were found.

In the three splenectomized dogs (Nos. 1, 3, and 7) the same changes were found in the remaining hemopoietic organs, but they were more marked. In fact, the greater pigment content of these organs, coupled with the greater urobilin excretion, suggests from another point of view Asher's (25) unconfirmed contention that the anemia of splenectomy is due to a failure to reutilize the products of destroyed erythrocytes. The relatively greater bone marrow hyperplasia in these animals has been described elsewhere (26) as occurring after splenectomy. It should be noted again, however, that this is not necessarily either wholly or in part a sign of increased hemopoiesis. In the only dog actually killed by transfusions, the signs of blood destruction were no greater, but congestion of all organs was most extreme. In fact, in the lungs, spleen, and lymph nodes, large areas of packed erythrocytes gave a picture indistinguishable from hemorrhage. It was not possible, however, to establish conclusively the immediate cause of death in this animal.

In the rabbits of this series, the outstanding feature was the widespread evidence of agglutination and thrombosis. Not only is the sudden death apparently to be explained by the extensive intravascular agglutination of blood cells and pigment, but also evidences of former thrombi are to be found within the blood vessels and in the focal necroses and fibroses of liver and spleen. The frequency of these agglutination thrombi, as well as the general frailty of the rabbit, militates strongly against its usefulness in this kind of work if experiments are to be continued over a long period. The same deposition of blood pigment in the various organs was found as in the dog, with one exception. In the rabbit, as in human hemochromatosis, the blood pigment is found in two forms: the iron-reacting granules of

hemosiderin, and minute semiopaque spicules that fail to react to the usual iron tests (probably hemofuscin). This latter pigment is especially prominent in the protoplasm of the liver cells (which in dogs were never found to contain hemosiderin), but is also found in large amounts in the various sites already mentioned.

DISCUSSION.

The relationship of artificial plethora to bone marrow activity as indicated by the percentage of reticulocytes, emphasized in the case of rabbits by Robertson (3), has been confirmed in these experiments both with dogs and rabbits. We have found that with the development of plethora the reticulocytes in the blood decreased, and that after anemia developed a reticulosis was manifest in every case. During recovery from the experimental anemia the reticulocytes were present in greatest numbers. This would indicate that while "living blood" was being supplied in excess from outside, the normal factory for erythrocytes suspended operation; the slow resumption of activity when erythrocytes were needed during the period of anemia (about 1 to 2 weeks) points either to Itami's inactivity atrophy, or to a bone marrow-inhibiting influence that required considerable time for removal.

The urobilin excretion, which under the constant conditions of these experiments can be taken as a reliable index of blood destruction, began to rise in the feces soon after transfusions were begun. It was greater during the "plethoric anemia" than in extreme plethora, but quickly lessened after transfusion was stopped. Although urobilin could not be detected in the urine during the early stages of plethora, it appeared there simultaneously with the occurrence of extreme blood destruction. This accords with the findings of other investigators that fecal urobilin may be greatly increased before it appears in the urine.

Details of the manner in which blood is normally destroyed in the body are still open to question. It was hoped that the addition of large quantities of blood and the subsequent destruction of this excess blood would disclose the underlying mechanism of blood destruction. Phagocytosis has been considered as a possible means of blood destruction, and the importance of endothelial cells in the spleen and the

Kupffer cells of the liver has been pointed out by Pearce and Austin (21). Rous and Robertson (24) found an appreciable phagocytosis in dogs, but believe this would not suffice for normal blood destruction. It is unquestionable, however, that greatly increased phagocytosis of blood pigment occurs in artificial plethora, as it does in many forms of increased blood destruction. The fragmentation of erythrocytes, which they consider an important factor in the destruction of blood, was not observed by us in the circulation in amounts greater than that seen under normal conditions. It is, of course, possible that as we did not enter into the special procedures that they undertook, this may have played a greater part than we appreciate. The relation of the postmortem findings to this topic has already been discussed.

It is well known that parenteral introduction of certain foreign substances will cause the formation of antitoxins, agglutinins, and hemolysins, and that antibodies may sometimes be formed after injection of protein from the same species. It is quite obvious that the first of these factors, namely antitoxins, may be eliminated in these experiments. As previously mentioned, Robertson (3) and Kambe and Komiya (4) have shown the presence of antibodies capable of destroying erythrocytes, even of the same species, in large numbers during plethora in rabbits; and isohemolysins and isoagglutinins have been produced in cats after compatible transfusions (5).

In a further attempt to explain the mechanism of this "plethoric anemia" by the production of agglutinins or hemolysins, ten rabbits were transfused by us with 5 to 20 cc. of compatible rabbit blood daily, according to the method previously described. Their blood changes corresponded in the main with those we have reported in dogs, but at no time were we able to demonstrate the presence of isoagglutinins or isohemolysins. Thinking that in our previous study on dogs, antibodies might have developed, but been absorbed by the daily repeated injections, we discontinued transfusions on the anemic rabbits for varying lengths of time before the antibody tests were made. Carefully controlled quantitative tests, with the complement fixation method, still continued negative. We are therefore unable to aid in the solution of this phase of the problem, and have no explanation as to why, like Boycott and Douglas and other observers, we were unable to demon-

strate either agglutinins or hemolysins while blood was obviously being destroyed in greatly increased quantities and in spite of the fact that in the rabbits agglutinative thrombi were found at autopsy. Faulty technique, the most obvious explanation, we endeavored to guard against most carefully. The ability of transfused rabbits to survive was extremely slight for reasons already discussed under the section on pathological anatomy.

In regard to the effect of splenectomy on plethora, and on the consequent anemia, one cannot but be impressed by the more constant and striking anemia of the dogs splenectomized during the course of the transfusions (Nos. 6 and 7) than of those that were not splenectomized (Nos. 4 and 5). In view of Hektoen's demonstration (27, 28) that under certain conditions antibody formation is lowered after splenectomy, and of our inability to demonstrate antibodies at any time, this anemia of splenectomized dogs can hardly be based on such grounds; but we feel confident that some relationship exists between the loss of the spleen and the marked anemia that developed. It is also of interest that "plethoric anemia" is harder to produce in dogs than in rabbits, animals whose spleens are relatively much smaller (0.05 per cent of body weight as against 0.25 per cent of dogs).

The two chief points of interest in the blood volume studies are: (1) the confirmation of previous findings as to the constancy of the plasma volume under widely varying conditions, and (2) the remarkable adaptability of the organism in accommodating itself to rapid increases in total blood volume reaching as high as 250 per cent of the normal.

The metabolism studies have pointed out a marked retention of nitrogen by the organism for a long period of time. We are not able to learn from these experiments how and when this excess nitrogen is eliminated from the body. This retention and the failure of the hemoglobin to rise appreciably during the first 10 days of transfusions with a corresponding lack of increased nitrogen elimination would seem to indicate the existence of a mechanism for the storage of considerable excess blood and its decomposition products.

The possibility of finding a true plethora followed by anemia in clinical medicine is illustrated by a recent report by Freund (29) of a case studied in Krehl's clinic.

After incurring syphilis and other infectious diseases, the patient developed a typical Vaquez polycythemia of 135 per cent hemoglobin, and blood count of 9,200,000 red cells. Several months later the blood picture gradually changed to that of pernicious anemia, with megaloblasts, stippled red cells, normoblasts, and urobilinuria. This condition was next followed by exhaustion of the hemopoietic system with absence of nucleated red cells, myelocytes, and platelets, a steadily falling hemoglobin, and finally death from aplastic anemia.

SUMMARY AND CONCLUSIONS.

1. The effects of repeated transfusions of blood on the blood-destroying and blood-forming apparatus of normal and splenectomized dogs and rabbits have been described. An anemia which developed despite continued blood transfusions in two dogs splenectomized during plethora has also been studied.

2. The decrease or absence of reticulocytes from the blood stream during plethora and their increase during "plethoric anemia" are evidently due to depression and activation of bone marrow activity. The response of the bone marrow is not immediate upon the onset of anemia, but is delayed for several days.

3. The blood volume studies have served to emphasize the constancy of plasma volume under extreme experimental conditions, and the adaptability of the circulatory system to large increases in total blood volume.

4. Blood destruction and elimination, as measured by urobilin excretion, are greatly increased during the stage of plethora, but still more so during "plethoric anemia."

5. Despite intravenous introductions of large quantities of nitrogen in the form of whole blood, the total nitrogen, urea, and ammonia in the urine and feces are not raised appreciably for some time after the onset of plethora. The normal organism is apparently able to store large quantities of blood or its decomposition products. Upon the onset of a "plethoric anemia," there is an increase in urinary total nitrogen and urea excretion, which was lowered during the course of the anemia. Albuminuria is also found at this time. Other nitrogenous constituents and phosphates show no striking changes.

6. Blood pigment, chiefly in the form of hemosiderin, is deposited in enormous quantities in the spleen, liver, lymph nodes, and bone marrow. It occurs chiefly in phagocytes, though in late stages large

extracellular masses are found. Increased pigment deposition can still be found several months after transfusions have been stopped.

7. Phagocytes containing erythrocytes are found rarely, if at all, and only in the acute cases, but their occurrence may be greatly masked by the coexistent congestion.

8. In splenectomized dogs the tendency to "plethoric anemia" is much more apparent, although a direct connection between the two events is not established. In rabbits, whose spleens constitute only 0.05 per cent of body weight, "plethoric anemia" is more easily produced.

9. In splenectomized animals pigment-bearing phagocytes are especially prominent in the liver, although lymph nodes and bone marrow apparently share in the extra work caused by the absence of the spleen. Lymph nodes with some of the characteristics of hemolymph nodes were found in various localities in all animals that had been made plethoric.

10. In rabbits blood pigment is deposited in the hemopoietic organs in large amounts, but under the conditions of our experiments, the picture and the experiment have been constantly complicated by early fatal intravascular agglutination and thrombosis. In the rabbit, as in human hemochromatosis, the pigment is found in two forms: hemosiderin granules, and smaller, dark spicules that do not react to the usual iron stains (probably hemofuscin). The latter pigment is also found seeded through the cells of the liver parenchyma.

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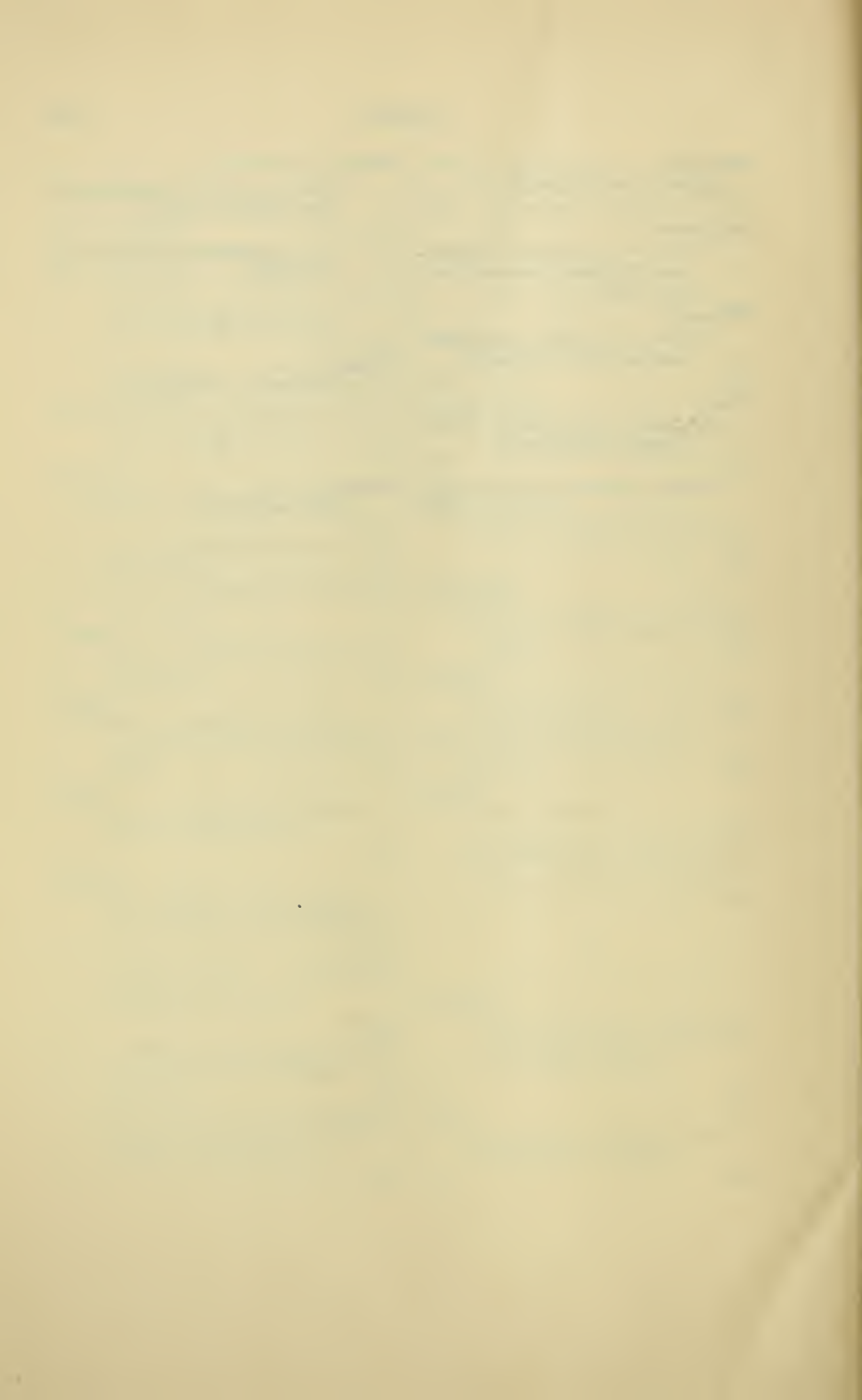
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